Structural and functional characterisation of avian IgY

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Structural and Functional Characterisation of Avian IgY

by

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Abstract

The principal serum antibody in birds, IgY, represents a conserved form of the last common ancestor of two important mammalian antibody isotypes, IgG and IgE. As IgG and IgE perform very different functions in the mammalian immune system, the structure and function of chicken IgY was studied to better understand the evolutionary changes that brought about this divergence.

Paradoxically, IgY appears to combine some of the structural and functional features of IgG and IgE in one molecule. IgY is functionally equivalent to IgG, mediating important immunological effector functions, including opsonisation, phagocytosis, complement activation and neonatal protection following transportation into the egg yolk.

Structurally, however, IgY shares features with IgE, the isotype responsible for the immediate hypersensitivity reaction which, as systemic anaphylaxis, is potentially fatal. Certain structural characteristics of IgE-Fc, particularly those shared by IgY but not IgG, are known to contribute directly to its anaphylactic properties by influencing the kinetics of binding to its Fc receptor, FcεRI, i.e. slow dissociation rate and long half-life of receptor-bound IgE in tissue.

A series of recombinant IgY-Fc fragments were designed and expressed, containing various mutations to explore the effects of glycosylation and inter-heavy chain disulfide bond linkage on the properties of IgY-Fc. Cells expressing Fc receptors were identified and used in cell-binding assays to characterise the interaction with IgY-Fc. Several features of IgY-Fc were found to be conserved

in IgG-Fc or IgE-Fc, and their structural and functional consequences were investigated, providing insights into the evolution of IgG and IgE.

Finally, two receptor sequences, chFcR/L and FcRγ, were cloned from chicken leukocytes and found to be homologous to important immunoregulatory receptor families in mammals. Unusual features of chFcR/L were identified, adding to the rapidly expanding body of research into the phylogeny of the adaptive immune system.

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Abbreviations

A	(Spectrophotometry) Absorbance
	(Electrophoresis) Amps
aa	Amino acids
(m)Ab	(Monoclonal) Antibody
AC	Affinity chromatography
ACD	Acid citrate dextrose
ampR	Ampicillin resistence gene
bp	Base pairs
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
C or CH	Constant heavy chain immunoglobulin domains
°C	Degrees Celsius
CD	(Receptors) Cluster of differentiation
	(Spectroscopy) Circular dichroism
ch	Chicken
cm	Centimetres
hCMV	Human cytomegalovirus immediate early promoter
cpm	Scintillation counts per minute
dCTP	Deoxycytidine triphosphate
Da	Daltons
DEAE	Diethylaminoethyl
DEPC	Diethylpyrocarbonate
(c)DNA	(Complementary) Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
DLS	Dynamic light scattering
ε	Epsilon heavy chain
E. coli	Escherichia coli
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
F	Farads

Fab	Antigen binding fragment of an antibody
FACS	Fluorescence activated cell sorter
dFBS	Dialysed foetal bovine serum
Fc	Receptor binding 'fragment crystallisable' of an antibody
FcεR	Fc receptor for IgE
FcγR	Fc receptor for IgG
FcR	Fc receptor
FcRγ	Fc receptor subunit gamma
FCRL	Fc receptor-like
FcRn	Neonatal Fc receptor
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
γ	Gamma heavy chain
g	(Mass) grams
	(Force) G-force
G/A	Glutamate / asparagine
GS	Glutamine synthetase
HPLC	High performance liquid chromatography
HRP	Horse Radish Peroxidase
hu	Human
^{125}I	Iodine-125
IAA	Isoamyl alcohol
IE	Ion exchange chromatography
Ig	Immunoglobulin
k	Kilo ($x10^3$)
k_a	Association rate constant
k_d	Dissociation rate constant
L	(Cell binding) Ligand
l	Litre
LB	Luria-Bertani
LRC	Leukocyte receptor complex
m	(Unit prefix) Milli ($x10^{-3}$)

	(Distance) Metres
M	Molar (moles per litre)
μ	(Unit prefix) Micro ($x10^{-6}$)
	(Protein) mu heavy chain
MCS	Multiple cloning site
MHC	Major histocompatibility complex
min	Minute
mol	Moles
Mwt	Molecular weight
mya	Millions of years ago
n	Nano ($x10^{-9}$)
NMR	Nuclear magnetic resonance spectroscopy
OD	Optical density
OPD	O-phenylenediamine dihydrochloride
^{32}P	Phosphorous-32
p	(Unit prefix) Pico ($x10^{-12}$)
	(DNA) Plasmid
PAS	Periodic acid-Schiff stain
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pIgR	Polymeric immunoglobulin receptor
PVDF	Polyvinylidene difluoride
R	(Cell binding) Receptor
RACE	Rapid amplification of cDNA ends
RT	Reverse transcription / transcriptase
RPE	R-phycoerythrin
rpm	Revolutions per minute
s	Seconds
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
SVE	SV40 early promoter

TBS	Tris-buffered saline
TE	Tris / EDTA
TEMED	N,N,N',N' tetramethylethylenediamine
T_m	Melting temperature
υ	Upsilon heavy chain
UTR or U	Untranslated region
UV	Ultraviolet light
V	Volts
wt	Wild type (i.e. unmutated)

Chapter 1

Introduction: IgY and the evolution of mammalian immunology

1.1 Overview: studying vertebrate evolution by comparison of extant species

Evolutionary biologists must piece together clues from the present in order to study the past. The rate of evolutionary change in a population of organisms is driven by selection pressure, which is unique to a particular ecological niche. Species that survive to pass on their genetic information through successive generations will remain well conserved in the absence of further impetus to adapt to their environment. One of the most famous examples of this are the coelacanths, a lobe-finned fish thought to be one of the closest extant relatives of all land vertebrates (Johanson *et al.*, 2006). Their modern-day counterparts, *Latimeria* (Smith, 1939), bear a striking resemblance to relatives preseved in 65-400 million year old fossils (Figure 1.1 i). Although the amount of time that has passed for the coelacanth lineage and for the lineage of modern mammals since their divergence has been the same, their rates of change have clearly been significantly different. In some instances, comparative biology can therefore allow deductions to be made about common ancestors which are now extinct. At the molecular level, this concept applies to sequence divergence of homologous genes. With some notable exceptions, biomolecules in fossilised remains older than the cenozoic have not survived to the present. Therefore, the only way to study divergence over a range of

evolutionary timescales is to compare present-day examples from different taxonomic classes (Hillier *et al.*, 2004).

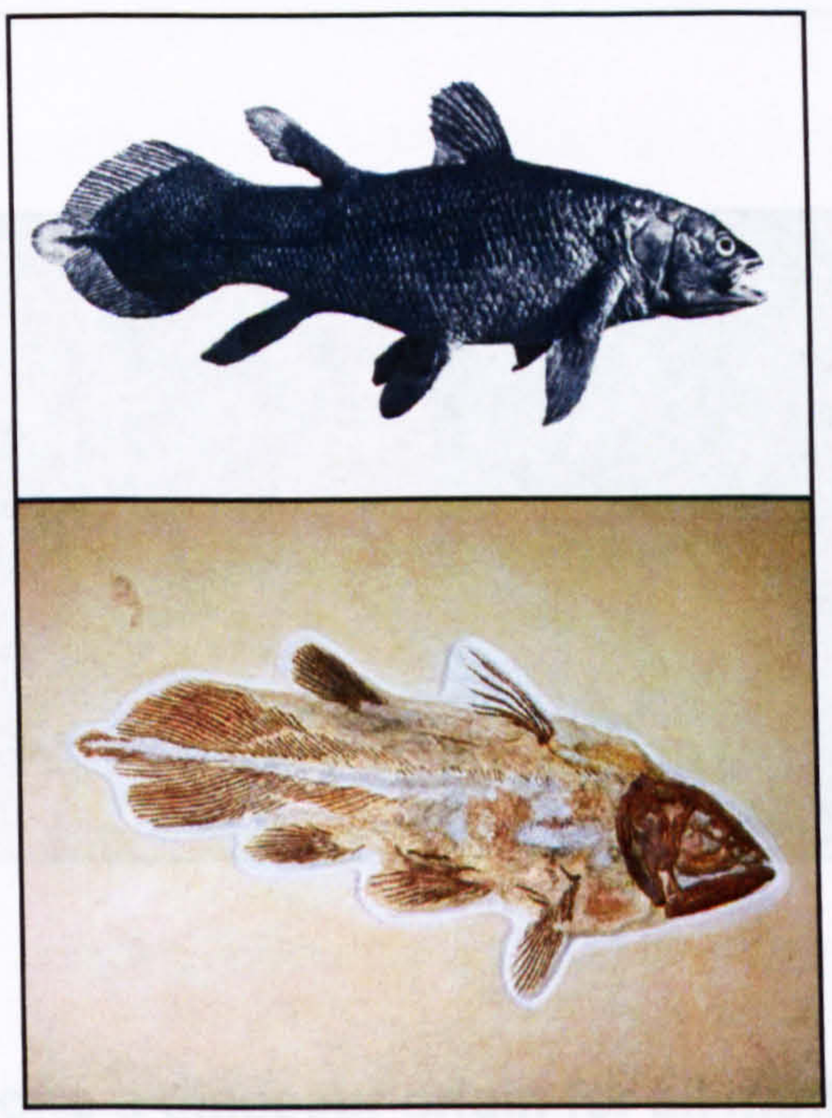


Figure 1.1 i
A living fossil: coelacanths are an extreme example of how evolution and selection pressure are inexorably linked

One of the greatest zoological 'discoveries' of the 20th century, a modern-day coelacanth, *Latimeria* (top), two species of which have now been described (Pouyaud *et al.*, 1999; Smith, 1939). Numerous coelacanths with astonishingly similar morphology have been unearthed as fossils (bottom), the oldest of which dates back ~400 million years (Johanson *et al.*, 2006).

Birds (aves) are one of the closest classes to mammals; the last common ancestor was thought to have lived ~310 million years ago (Hedges, 2002; Reisz and Muller, 2004), which places birds at a useful 'intermediate' phylogenetic distance from mammals in the vertebrate timescale (Furlong, 2005). The avian fossil record is patchy, but includes *Archaeopteryx*, a ~150

million year old relative of all extant bird species, including chickens (Figure 1.1 ii). *Archaeopteryx* (and other primitive birds) combine features of both dinosaurs and birds, which supports a theropod origin for all modern day birds (Mayr *et al.*, 2005).

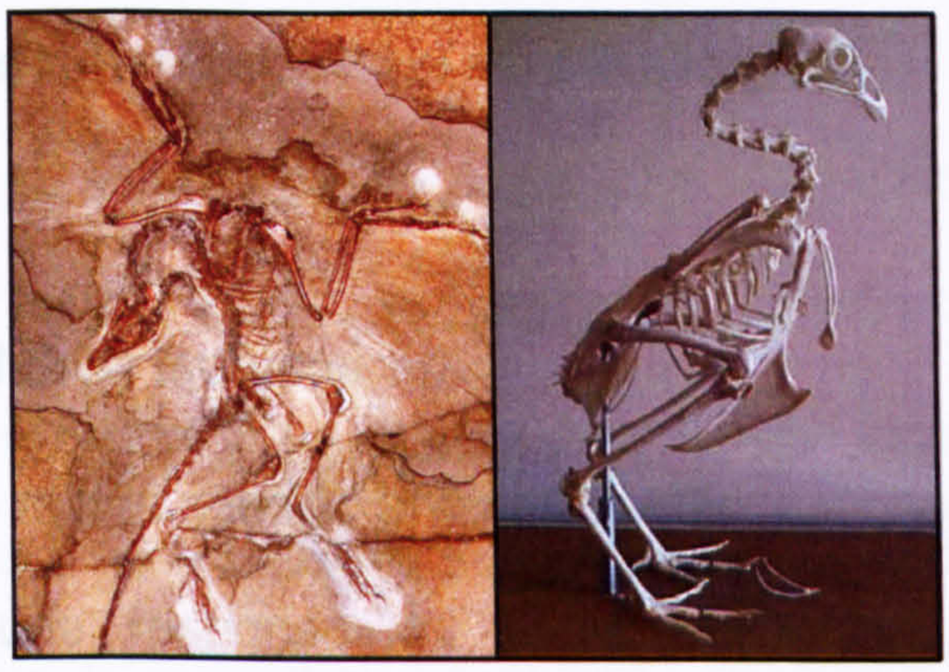


Figure 1.1 ii
***Archaeopteryx* supports a dinosaur origin for all modern birds**
The first *Archaeopteryx* fossil (left), discovered in 150 million year old limestone in Solnhofen, Germany, appears to share features with both dinosaurs and modern-day birds, such as the chicken (right).

Molecular evidence in agreement with this classification is beginning to be elucidated (Vargas and Fallon, 2005), although debate as to when speciation of most bird lineages occurred (the 'neoaves radiation') continues (Brown *et al.*, 2007; Ericson *et al.*, 2006; Ericson *et al.*, 2007). Amazingly, *Tyrannosaurus* collagen was recently isolated from a uniquely well preserved cretaceous fossil by Schweitzer *et al.* and, consistent with the relationship between dinosaurs and birds, the protein sequence was found to share highest identity with a chicken homologue (Asara *et al.*, 2007; Schweitzer *et al.*, 2007). The fossil also

appeared to contain red blood cells, which were nucleated as they are in birds (Schweitzer *et al.*, 2005).

The overall aim of the work presented in this thesis is to exploit the phylogenetic relationship between chickens (*Gallus gallus*) and humans to gain a better appreciation of the evolutionary history of some important antibody isotypes, with particular emphasis on IgE, the mammalian isotype typically responsible for priming of immediate hypersensitvity in severe allergics.

1.2 Antibodies and their isotypes

The ability to mount specific anti-pathogenic responses is the hallmark of the adaptive immune response. In jawed vertebrates, lymphocytes that recognise a particular antigen, following generation of receptor diversity through a variety of techniques (Cooper and Alder, 2006), can become activated to secrete antibodies (immunoglobulins). This strategy allows gene rearrangements (and/or mutations) necessary to generate antigen receptor diversity to occur in a particular set of immune cells. The resulting ability to recognise specific antigen is thus disseminated to a variety of cell types that express Fc receptors (FcR), thereby linking humoral and cellular immunity. Through precisely regulated expression of antibodies and their Fc receptors, the immune system is capable of tailoring where and how it responds to a particular threat (Ravetch and Kinet, 1991).

Antibodies are glycoproteins and most share a similar heterotetrameric structure of two heavy and two light chains composed of globular domains containing the immunoglobulin (Ig) fold (Amzel and Poljak, 1979), one of the most abundant motifs in the animal kingdom due to its evolutionary mobility

(Doolittle and Bork, 1993). The heavy and light chains are joined by disulfide and non-covalent bonds in such a way that three separate regions are formed (Figure 1.2): two identical Fab arms, which contain the variable regions that form the antigen binding site, and an invariant Fc region, which contains binding sites for cellular activatory and inhibitory receptors, soluble regulatory receptors and the complement system (Sondermann and Oosthuizen, 2002; Woof and Burton, 2004).

In mammals, there are 5 major antibody isotypes (IgM, IgD, IgG, IgE, IgA), distinguished by structural and functional differences in their Fc regions; classically, the molecular weight of their heavy chains (Janeway, 2001), and the particular Fc receptors by which they are recognised (Ravetch and Kinet, 1991). In humans there are four subclasses of IgG, which vary in the length of a flexible hinge connecting their Fab and Fc regions, although it is IgG1 that occurs at the highest levels in serum, ~9mg/ml.

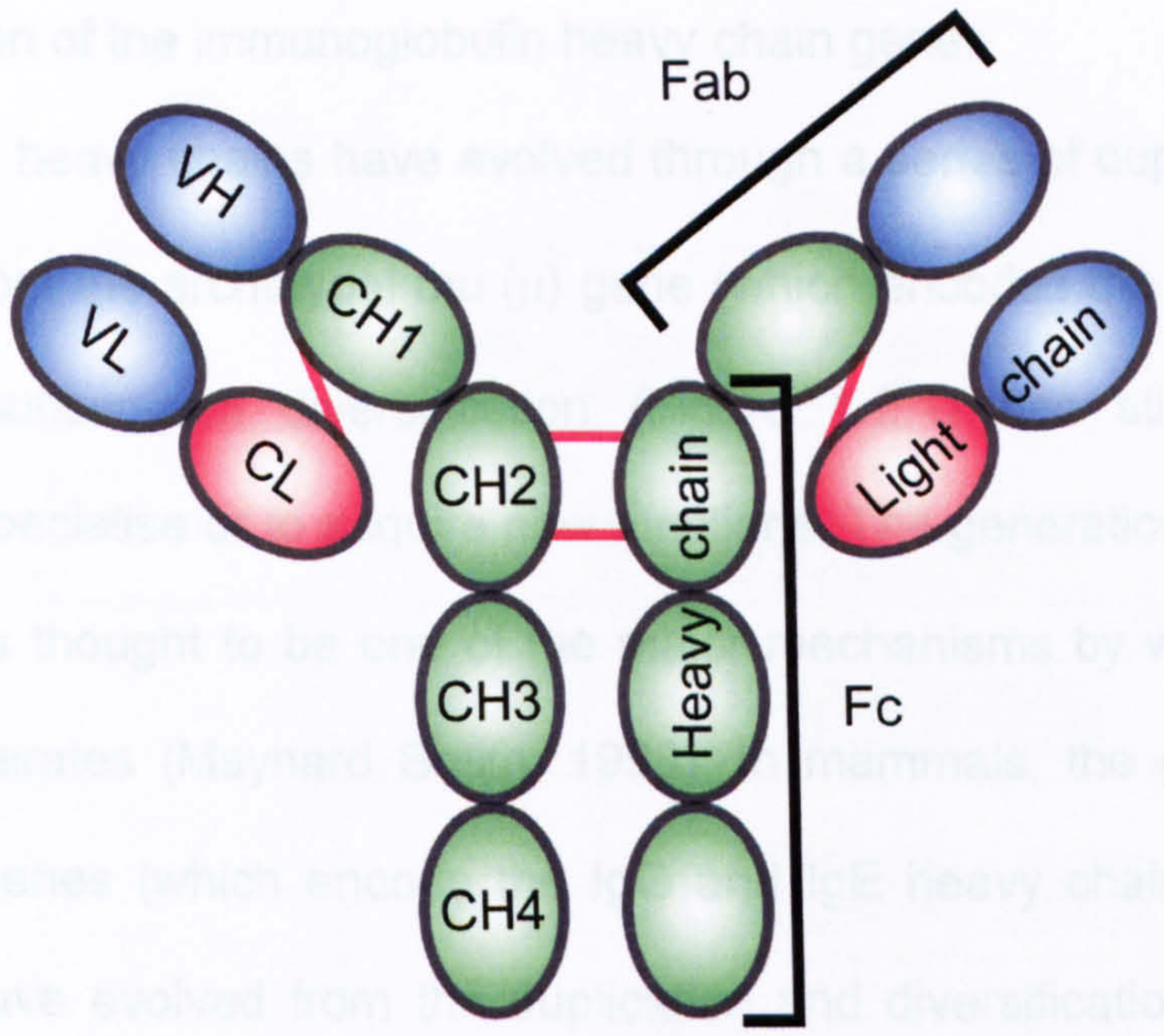


Figure 1.2
Typical antibody (immunoglobulin) architecture

Schematic showing the basic structural organisation of an antibody molecule. Two heavy (H) and two light (L) chains, composed of either variable (VH or VL) or constant (CH or CL) immunoglobulin domains, are held together by disulfide bonds (shown in red). In IgG, IgD and IgA a hinge is in place of CH2. Fab and Fc regions are indicated.

In birds, reptiles and amphibians, the predominant serum immunoglobulin is an isotype distinct from IgG, called IgY (Amemiya *et al.*, 1989; Chartrand *et al.*, 1971; Fellah *et al.*, 1993; Leslie and Clem, 1969; Leslie and Clem, 1972; Magor *et al.*, 1992). IgY has the typical heterotetrameric structure of other antibody isotypes (i.e. the overall architecture of Figure 1.2), but is distinct from mammalian IgG, which contains a hinge linker region instead of one of the Ig domains (that labelled CH2 in Figure 1.2) in each of its heavy (upsilon, υ) chains. This, and other structural features, suggest that IgY is more similar to mammalian IgE than IgG (Parvari *et al.*, 1988).

1.2.1 Evolution of the immunoglobulin heavy chain genes

The Ig heavy chains have evolved through a series of duplication events originating from the archetypal mu (μ) gene (which encodes the heavy chain of IgM), and subsequent diversification (Figure 1.2.1) has allowed different isotypes to specialise or to acquire new functions. The generation of paralogues in this way is thought to be one of the major mechanisms by which molecular evolution operates (Maynard Smith, 1998). In mammals, the gamma (γ) and epsilon (ε) genes (which encode the IgG and IgE heavy chains respectively) appear to have evolved from the duplication and diversification of an upsilon (υ)-like common ancestor (Warr *et al.*, 1995; Flajnik, 2002). Consequently, the adaptive immune systems of modern day mammals now use IgG and IgE in place of IgY, whereas the ancestral isotype has been conserved in the amphibian, reptile and bird lineages.

Egg-laying mammals (monotremes), the most 'primitive' extant mammal order, possess IgG and IgE that have the same structural organisation as their placental and marsupial homologues (Atwell and Marchalonis, 1977; Vernersson *et al.*, 2004; Vernersson *et al.*, 2002). The most recent phylogenetic analysis suggests that monotremes diverged from other mammals ~166 million years ago (Bininda-Emonds *et al.*, 2007), so the duplication of υ and the divergence to γ and ε must have occurred in a relatively narrow window (by evolutionary standards) of 166-310 million years ago.

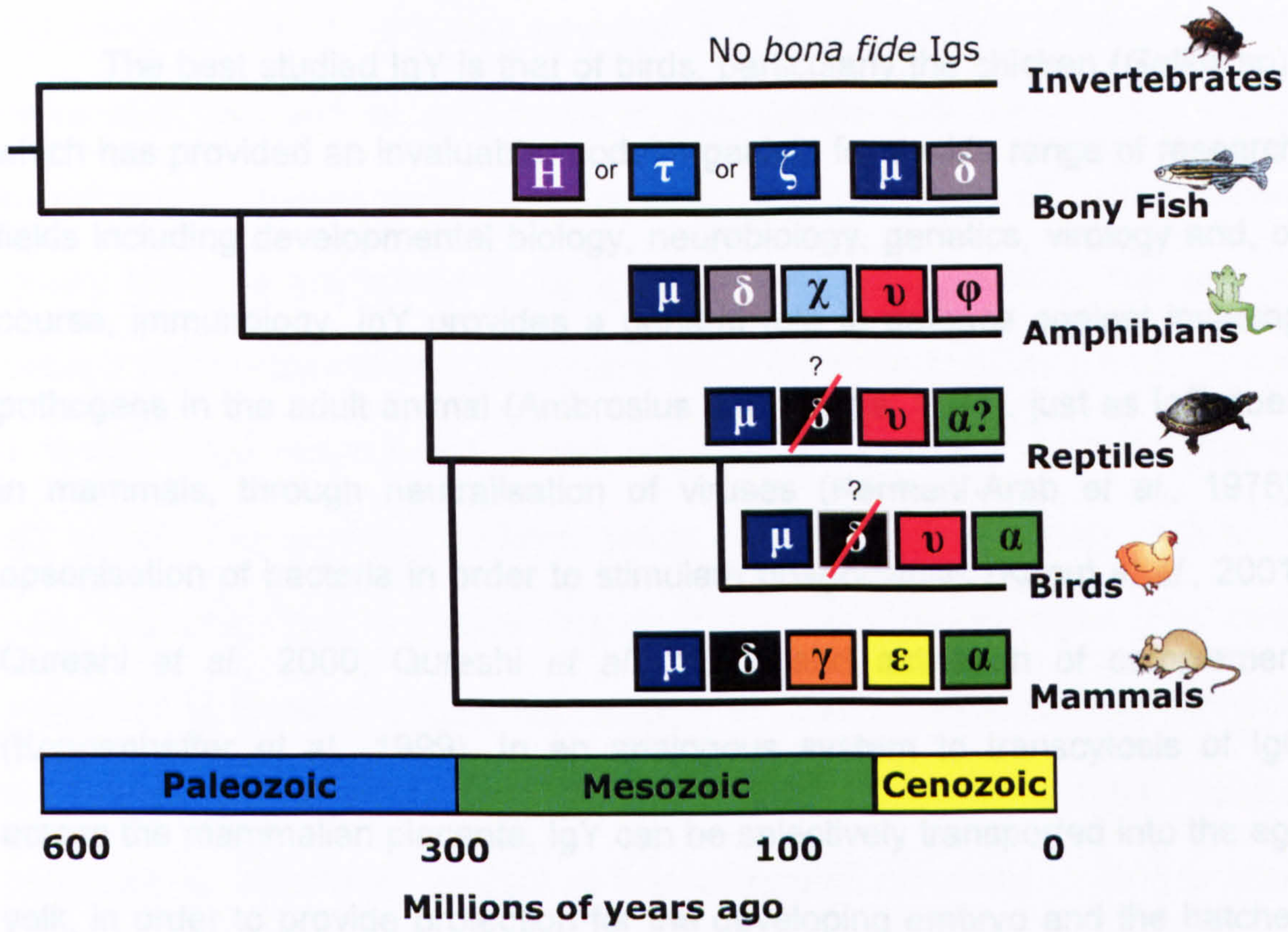


Figure 1.2.1
Phylogeny of the immunoglobulin heavy chains

Phylogram showing the appearance of immunoglobulin heavy chain genes (and therefore Ig isotypes) throughout the jawed vertebrates. IgM (μ) occurs in all gnathostomata analysed to date and is thought to be ancestral to all other isotypes, which have been derived through gene duplication events (Flajnik, 2002). Several isotypes have arisen in a lineage-restricted fashion or have been lost in the common ancestors of other lineages: teleost IgZ (ζ) (Danilova *et al.*, 2005), IgH (H) (Savan *et al.*, 2005), IgT (τ) (Hansen *et al.*, 2005) and amphibian IgF (φ) (Zhao *et al.*, 2006) and IgX (χ) (Hsu *et al.*, 1985). IgY (υ) appears to be well conserved in amphibians (Amemiya *et al.*, 1989), reptiles (Chartrand *et al.*, 1971; Fellah and Charlemagne, 1988) and birds (Magor *et al.*, 1992; Parvari *et al.*, 1988) but appears to have been duplicated in the mammal lineage to produce two new isotypes, IgG (γ) and IgE (ε) (Mussmann *et al.*, 1996; Warr *et al.*, 1995).

1.3 The nature of avian IgY and its mammalian homologues, IgG and IgE

The best studied IgY is that of birds, particularly the chicken (*Gallus* sp), which has provided an invaluable model organism for a wide range of research fields including developmental biology, neurobiology, genetics, virology and, of course, immunology. IgY provides a general role in defence against invading pathogens in the adult animal (Ambrosius and Hadge, 1987), just as IgG does in mammals, through neutralisation of viruses (Kermani-Arab *et al.*, 1976), opsonisation of bacteria in order to stimulate phagocytosis (Kogut *et al.*, 2001; Qureshi *et al.*, 2000; Qureshi *et al.*, 1990) and activation of complement (Koppenheffer *et al.*, 1999). In an analogous system to transcytosis of IgG across the mammalian placenta, IgY can be selectively transported into the egg yolk, in order to provide protection for the developing embryo and the hatched chick (Linden and Roth, 1978; Malkinson, 1965; Tressler and Roth, 1987; West *et al.*, 2004).

IgY has been shown to be capable of mediating passive cutaneous anaphylaxis (Bellavia *et al.*, 1992; Chand *et al.*, 1976; Faith and Clem, 1973) through sensitisation of granulocytes (Wilson and Heller, 1976) and has been shown to be involved in mast cell activation in the chicken gut in response to protozoan infections (Rose *et al.*, 1980). In mammals, these properties are normally associated with IgE, the isotype that sensitises mast cells and basophils in classic type I hypersensitivity (Gould *et al.*, 2003), although murine studies have suggested that IgG may also be capable of inducing anaphylactic responses, albeit in very high concentrations (Finkelman *et al.*, 2005; Woolhiser *et al.*, 2001).

IgY was originally referred to as chicken IgG, or γ-globulin (Dreesman and Benedict, 1965a), but was renamed due to its structural differences with mammalian IgG and its occurrence in yolk (Leslie and Clem, 1969). cDNA encoding the chicken upsilon heavy chain has been cloned, which allows tertiary structure predictions to be made (Parvari *et al.*, 1988). The gamma heavy chain of mammalian IgG contains three constant domains (Cγ1 to 3) with a flexible hinge region between Cγ1 and Cγ2, whereas both IgE and chicken IgY contain four constant domains (each encoded by a separate exon as in other isotypes) and no hinge (Figure 1.3 i). The hinge region has been suggested to increase segmental flexibility in order to allow the Fab 'arms' to engage antigen more effectively (Nezlin *et al.*, 1973; Oi *et al.*, 1984), so may represent an adaptation of IgG to immunological demands to bind a more diverse range of multivalent antigens. The presence of IgF, a hinge-containing isotype related to IgY in an amphibian, *Xenopus tropicalis* (Zhao *et al.*, 2006), suggests that this modification of IgY has evolved at least twice, which probably reflects its immunological usefulness.

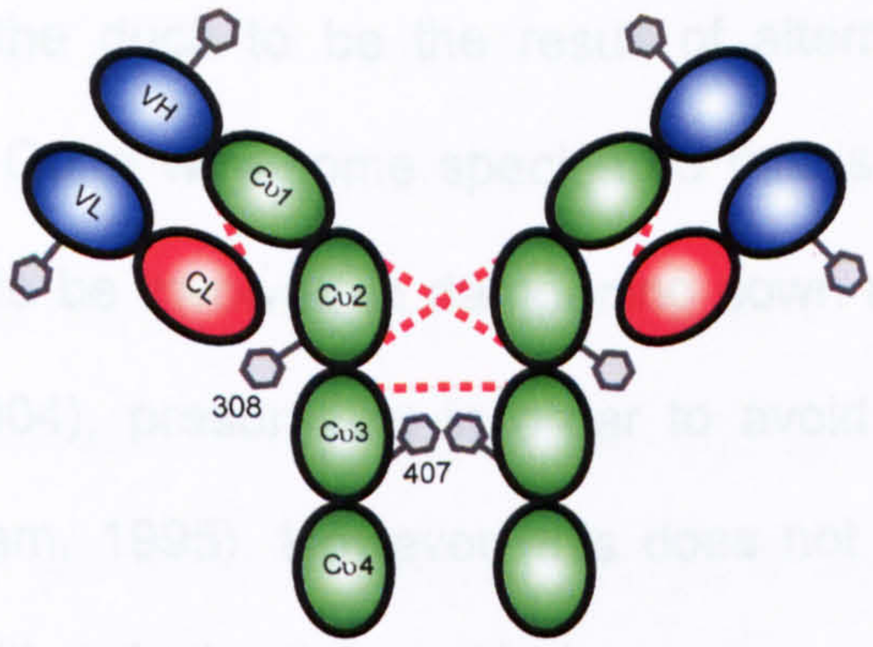


Figure 1.3 i
Structural similarities between chicken IgY and its mammalian homologues, IgG and IgE

Schematic showing predicted structure of chicken IgY compared with human IgE and IgG1. IgG contains only three heavy chain constant domains, and thus Cγ2 and Cγ3 are homologous to Cυ3 and Cυ4 of IgY, respectively. The pattern of putative disulfide bonds and N-linked glycosylation in the Fc region in IgY is more similar to that of IgE than IgG.

Interestingly, although chicken produces only 'full length' IgY either in a secreted or membrane bound form (in its role as a B cell receptor), some species of amphibian (Marchalonis and Edelman, 1966), reptile (Chartrand *et al.*, 1971) and bird, particularly waterfowl (Lundqvist *et al.*, 2006), produce 'full length' IgY and/or a truncated version, IgY ΔFc, whose heavy chain lacks the last two C-terminal domains, Cυ3 and Cυ4 (Magor *et al.*, 1992); the ΔFc forms consequently have limited effector functions (Grey, 1967a; Grey, 1967b). This

has been shown in the duck to be the result of alternative mRNA splicing (Magor *et al.*, 1994). Quite why some species do this is poorly studied, but it has been suggested to be involved in dampening down acute phase reactions (Humphrey *et al.*, 2004), presumably in order to avoid IgE-like anaphylactic consequences (Parham, 1995). However, this does not explain how chickens are able to function with only the 'full length' form.

Many of the cysteine residues found in the upsilon heavy chain are conserved in both mammalian heavy chain classes, which implies similarities in disulfide bond configuration, although several of these are conserved between IgY and IgE, but not IgG. Typically, immunoglobulin domains contain a single 'intradomain' disulfide bond. Although this is true of most mammalian IgG Cγ1 domains, except that of rabbit (O'Donnell *et al.*, 1970), the equivalent domain in IgE, Cε1, contains two (Dorrington and Bennich, 1978). The Ig domains of the upsilon heavy chain contain the necessary cysteines at the appropriate positions for single intra-domain disulfides, although the homologous domain to Cε1, Cυ1, also contains an additional pair. In IgE, this trait and the position of interchain disulfides between the two heavy chains in Cε2 (Wan *et al.*, 2002) and between the heavy and light chains, appears to be inherited from its IgY-like precursor. In human IgG1, both the inter-heavy-light chain bond and the inter-heavy chain bonds are in different positions (Milstein and Pink, 1970). The upsilon heavy chain additionally contains another cysteine not found in the epsilon heavy chain (Figure 1.3 ii), which might allow the formation of a third interchain disulphide bond.

Further structural similarities between IgY and IgE concern the pattern and type of N-linked glycosylation. Although IgE is more heavily glycosylated in

the Fab region than IgY, their Fc regions share well-conserved glycosylation sites. The glycosylation site in chicken Cυ2 (N308) is equivalent to the site in Cε2 (see Figure 1.3 ii), but no homologous site exists in IgG. Although N407 of IgY is a site well-conserved in both IgE (N394) and IgG (N297), it is linked to high mannose in both IgE and IgY, whereas in IgG complex-type carbohydrate is attached (Suzuki and Lee, 2004).

```
epsilon     -ASTQSPSVFPLTRCCKNIPSNATSVTLGCLATGYFPEPVMVTWDTGSLNGTTMTLPATT 179
upsilon     ASPTSPPRLYPLSACCS---DSAVPPAVGCLLS---------PSSAGGISWEGSGGTAVA 190
             :.*..* ::**: **.   ..*..::*** :         . .:*.:.     .*.:

epsilon     LTLSGHYATISLLTVSGAWAKQMFTCRVAHTPSSTDWVDNKTFSVCSRDFTPPTVKILQS 239
upsilon     GRVSGTPVKLSFVRLSPGEKRKSFVCSAAPG----GALLKKEVQVCRVDPVPPVAPEVQV 246
              :**  ..:*:: :* .  :: *.* .*      . : :* ..**  * .**..  :*

epsilon     SCDGGGHFPP--TIQLLCLVSGYTPGTINITWLEDGQVMDVDLSTASTTQEGELASTQSE 297
upsilon     LHPSSCTPSQSESVELLCLVTGFSPASAEVEWLVDGVGGLLVASQSPAVRSGSTYSLSSR 306
              ..   .   :::*****:*::*.: :: ** **    :  * :.:.:.*.  * .*.

epsilon     LTLSQKHWLSDRTYTCQVTYQG--HTFEDSTKKCAD--SNPRGVSAYLSRPSPFDLFIRK 352
upsilon     VNVSGTDWREGKSYSCRVRHPATNTVVEDHVKGCPDGAQSCSPIQLYAIPPSPGELYISL 366
            :.:* ..* ..::*:*:* : .   ..** .* *.*  ..   :. *   *** :*:*

epsilon     SPTITCLVVDLAPSKGTVNLTWSRASGKPVNHSTRKEEKQRNGTLTVTSTLPVGTRDWIE 412
upsilon     DAKLRCLVVNLP-SDSSLSVTWTREKSGNLRPDPMVLQEHFNGTYSASSAVPVSTQDWLS 425
            ...: ****:*. *..::.:**:* ..  :. ..   ::: *** :.:*::**.*:**:.

epsilon     GETYQCRVTHPHLPRALMRSTTKTSGPRAAPEVYAFATPEWPGSRDKRTLACLIQNFMPE 472
upsilon     GERFTCTVQHEELPLPLSKSVYRNTGPTTPPLIYPFAPHPEELSLSRVTLSCLVRGFRPR 485
            ** : * * * .** .* :*. :.:** :.* :*.**.     * .: **:**::.* *.

epsilon     DISVQWLHNEVQLPDARHSTTQPRKTK---------GSGFFVFSRLEVTRAEWEQKDEFI 523
upsilon     DIEIRWLRDHRAVPATEFVTTAVLPEERTANGAGGDGDTFFVYSKMSVETAKWNGGTVFA 545
            **.::**::.  :* :.. **   :          *. ***:*::.*  *:*:    *

epsilon     CRAVHEAASPSQTVQRAVSVNPGK 547
upsilon     CMAVHEAL-PMRFSQRTLQKQAGK 568
            * *****  * :  **::. :.**
```

Figure 1.3 ii

Alignment of the heavy chain constant regions of human IgE and chicken IgY

Amino acid sequence alignment of constant regions of human epsilon heavy chain and chicken upsilon heavy chain. Immunoglobulin domains are differentiated by colour. Cysteine residues are highlighted as follows: Pink = forms inter-heavy-light chain disulphide bond. Blue = forms inter-heavy chain disulphide bond. Uncoloured = involved in intradomain disulphide bond. The additional cysteine in the upsilon chain (C347) that does not occur in the epsilon chain is shown highlighted in red. N-linked glycosylation sites are shown in grey.

The Cε2 glycosylation site in the human epsilon heavy chain (N265) is ~34 amino acids closer to the C-terminus in all other mammalian epsilon heavy chains sequenced to date (Vernersson *et al.*, 2004), a position which aligns with chicken N308 (Suzuki and Lee, 2004). The N407 core glycosylation site in the chicken upsilon heavy chain is well-conserved in both the epsilon heavy chain of IgE (N394) and the gamma heavy chain of IgG (N297, not shown).

Sequences were aligned using ClustalW (Thompson *et al.*, 1994). Accession numbers for sequences used: human epsilon heavy chain: L00022 (Flanagan and Rabbitts, 1982); chicken upsilon heavy chain: X07174 (Parvari *et al.*, 1988). See appendix 1 for an explanation of upsilon heavy chain amino acid numbering.

The sequence identity between Cυ, Cε and Cγ domains (up to ~35%) is consistent with the level seen between many bird/mammal homologues (Hillier *et al.*, 2004), particularly those of the immune system, one of the most rapidly evolving biological systems due to the selective pressure of the host/pathogen arms race (Hughes, 1997; Murphy, 1993). Comparison of υ, ε and γ heavy chain sequences does not unequivocally prove the basal relationship of IgY to IgG and IgE, even when individual domains are aligned, which Parvari *et al* ascribe to the divergence time between birds and mammals (Parvari *et al.*, 1988).However, recent phylogenetic analyses using heavy chain sequences of most isotypes cloned from a wide range of taxonomic classes support the proposed relationship (Vernersson *et al.*, 2004; Warr *et al.*, 1995).

Levels of homology between υ, ε and γ vary along the length of the heavy chain (see appendix 1) (Parvari *et al.*, 1988). CH1 is the most poorly conserved between υ/ε and υ/γ, and both Cυ2 and Cυ3 show similar amino acid identity with all three Cγ domains. Overall, domain equivalence seems to be most consistent between υ and ε. DNA segments and even whole exons (which encode a complete Ig domain) appear to have been reshuffled between the heavy chains of IgG subclasses in some mammals, either by gene conversion or unequal crossing-over (Hayashida *et al.*, 1984). Although this is thought not to have occurred between the human IgG subclasses, similar processes may have occurred early on in the mammal lineage, before the further duplication of the early γ gene to produce multiple subclasses.

1.4 The structure of IgE and its role in immediate hypersensitivity

IgE-mediated responses are thought to have developed in order to provide powerful anti-parasitic immunity (Capron *et al.*, 2004; Zaccone *et al.*, 2006), although they have been extensively studied due to their inappropriate role in allergy or type I hypersensitivity (Gould *et al.*, 2003).

Mast cells and basophils become primed when antigen-specific IgE binds to high affinity receptors on the cell surface. In this state, exposure to that antigen (the 'allergen') causes cross-linking of IgE, resulting in an activation signal to release inflammatory mediators that cause the sudden onset of allergic symptoms. Depending on many factors, such as genetic predisposition, allergen dose and mode of entry to the body, these symptoms can range from minor local effects to systemic anaphylaxis, which may be life-threatening (Gould *et al.*, 2003). Although many factors contribute to priming of the allergic response, IgE sensitisation of mast cells and basophils is a crucial part of the pathway and is primarily due to the uniquely high affinity of the interaction between IgE and its 'high-affinity receptor', FcεRI (Gould *et al.*, 1991).

IgE has an affinity (K_A) for FcεRI of ~10^{10} M^{-1}, which is approximately two orders of magnitude greater than the highest affinity IgG receptor interaction (Ravetch and Kinet, 1991) and five orders of magnitude greater than the affinity of IgG for FcγRIII, the receptor that is the closest relative of FcεRI (Maenaka *et al.*, 2001). This exceptionally high receptor affinity is predominantly caused by a slow rate of dissociation, leading to a half life of ~16 hours (McDonnell *et al.*, 2001), which is increased in tissue to ~2 weeks due to rebinding and slow antibody clearance (Geha *et al.*, 1985; Gould *et al.*, 2003). The half-life of

equivalent IgG:FcγR complexes, by contrast, is in the minute timescale despite structurally similar interactions (Garman *et al.*, 2000; Sondermann *et al.*, 2000).

Although IgE binds with high affinity through sites in Cε3 (Garman *et al.*, 2000), biophysical studies involving IgE-Fc fragments and soluble FcεRIα receptor domains have shown that the Cε2 domain is responsible for an increased stability of the IgE:FcεRI complex (McDonnell *et al.*, 2001). Interactions between a soluble Cε2 domain and both a soluble FcεRIα construct and a Cε3-4 fragment have been demonstrated using NMR and analytical ultracentrifuge experiments, respectively (McDonnell *et al.*, 2001). The crystal structure of the complete IgE-Fc region (Cε2-4) shows that it has an asymmetrical, acutely bent structure (Figure 1.4), with the Cε2 domains doubled back over in contact with one of the Cε3 domains (Wan *et al.*, 2002). These data, together with differences observed in the crystal structures of unbound Cε3-4 (Wurzburg *et al.*, 2000) and Cε3-4 in complex with receptor (Garman *et al.*, 2000) has led to the postulation of a conformational change upon FcεRI binding, which may explain the importance of Cε2 in determining the observed slow rate of dissociation that leads to effector cell priming *in vivo* (Wan *et al.*, 2002; Wurzburg and Jardetzky, 2002).

Accessibility of residues in the receptor binding site appear to be somewhat restricted in the unbound Cε3-4, with the Cε3 domains adopting a 'closed' conformation. In the bound structure, the orientation of the Cε3 domains has changed so that they are markedly further apart, or 'open'. In the complete (unbound) IgE-Fc structure, one of the Cε3 domains is in the 'open' state, but the other is 'closed'. A movement of at least one Cε3 domain therefore seems

necessary to allow full access to the receptor binding site, accompanied by a major rearrangement of the Cε2 domains, in order to expose the second Cε3 and to form an additional, stabilising interaction with the receptor (Wan *et al.*, 2002).

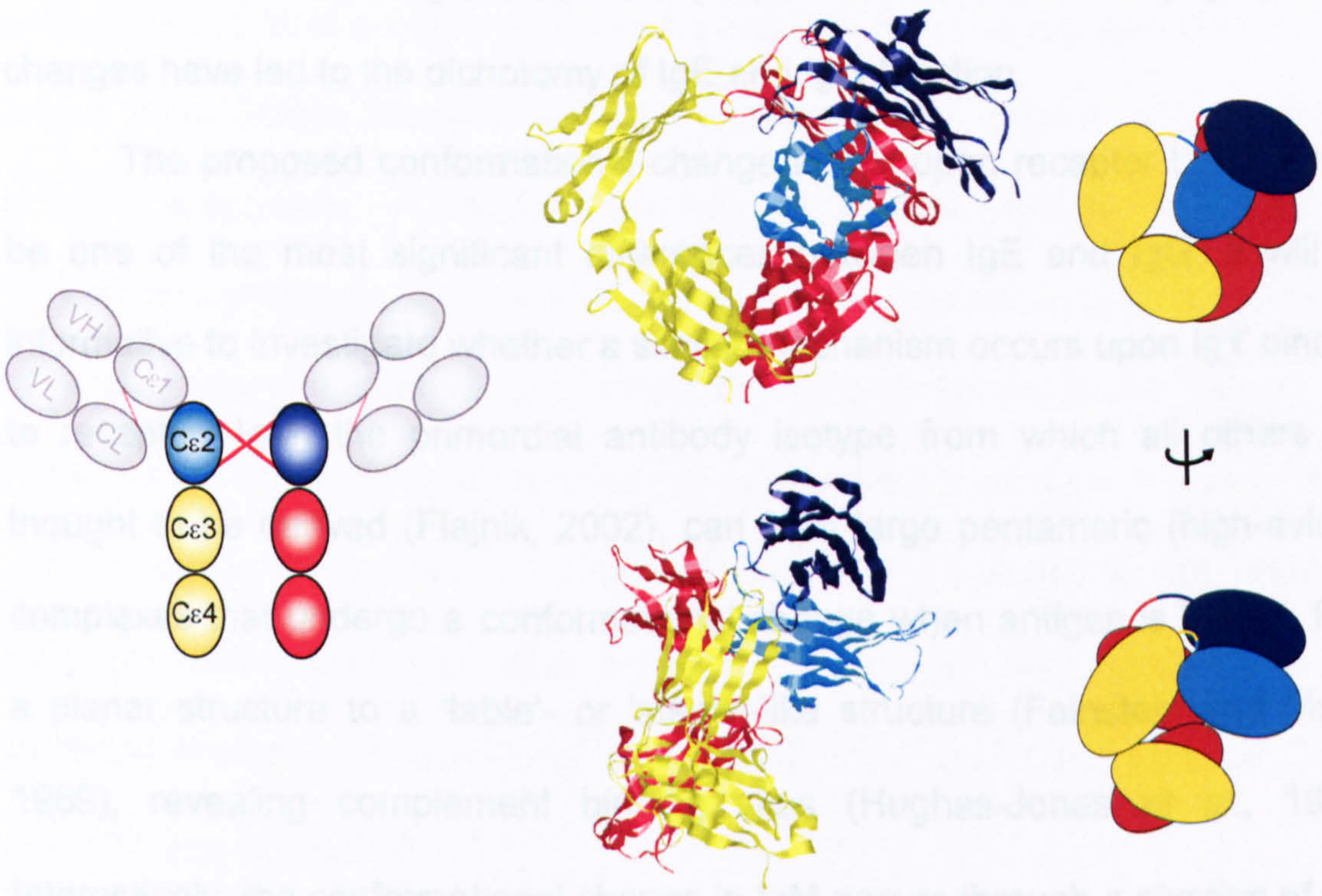


Figure 1.4
The structure of IgE-Fc is asymmetric and acutely bent

The crystal structure of full length IgE-Fc Cε2-4 solved by Wan *et al.* (2002) shows that whilst the Cε3-4 portion of the Fc has a symmetrical 'horseshoe' structure similar to that of IgG-Fc (Deisenhofer, 1981), the Cε2 domain pair is bent back over one of the Cε3 domains, not raised above them in the same plane, as predicted by previous models (Padlan and Davies, 1986). The crystal structures are shown in the ribbon diagram (centre), with schematics showing how domains are coloured (left) and oriented (right).

The lack of a domain homologous to Cε2 in IgG subclasses would preclude such a mechanism in this isotype, and may explain, or at least contribute to, the lower affinity of IgG-Fc receptor interactions. However, the apparent combination of IgG-like function and IgE-like structure in a conserved form of their evolutionary precursor, IgY, seems paradoxical. Further study of the structure and function of avian IgY and its Fc receptors in comparison with the mammalian homologues has the potential to reveal which phylogenetic changes have led to the dichotomy of IgE and IgG function.

The proposed conformational change in IgE upon receptor binding may be one of the most significant differences between IgE and IgG; it will be informative to investigate whether a similar mechanism occurs upon IgY binding to receptor. IgM, the primordial antibody isotype from which all others are thought to be derived (Flajnik, 2002), can form large pentameric (high-avidity) complexes that undergo a conformational change when antigen is bound, from a planar structure to a 'table'- or 'staple'-like structure (Feinstein and Munn, 1969), revealing complement binding sites (Hughes-Jones *et al.*, 1984). Interestingly, the conformational change in IgM occurs through a pivoting of Cμ2 (Perkins *et al.*, 1991), the domain homologous to Cυ2 in IgY and Cε2 in IgE. As the mu heavy chain of IgM was duplicated to produce the upsilon heavy chain of IgY, from which IgE was derived in mammals (Warr *et al.*, 1995), flexibility may have been conserved from Cμ2 to Cε2, via Cυ2.

1.5 Antibody Fc receptors and their homologues

Fc receptors have been studied extensively in mouse and human, although orthologues have been identified in many other mammals. Very little is known about Fc receptors in other vertebrate classes, but the existence of analogues can be inferred in species whose humoral immunity has similar aspects to that of mammals. Fc receptors provide the means by which antibodies are 'sensed' by the immune system in order to regulate their levels, export them across mucosal epithelia and, of course, trigger anti-pathogenic effector responses. Antibody-dependent responses include opsonisation-induced phagocytosis, antibody-mediated cellular cytotoxicity (ADCC), release of inflammatory mediators and classical complement-mediated killing. The nature of the antibody-induced response depends on a complex interplay of the various antibody isotypes with several types of Fc receptor that differ in structure, distribution, signalling potential, and affinity and specificity of the antibody interaction (Cohen-Solal *et al.*, 2004; Nimmerjahn and Ravetch, 2006; Ravetch and Kinet, 1991).

With a few exceptions, such as the low affinity receptor for IgE (FcεRII or CD23), which is a C-type lectin (Gould *et al.*, 1991) and the neonatal Fc receptor (FcRn), which is an MHC Class I related protein (Ghetie and Ward, 2000), the mammalian Fc receptors (FcR) for IgE and IgG are members of a closely related protein family which, like their ligands, are also members of the immunoglobulin superfamily. The 'α-(alpha) chains' of the receptors for the IgG subtypes, FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and the high affinity receptor for IgE, FcεRI are composed of two or three immunoglobulin-like domains, a transmembrane domain and a cytoplasmic tail (Table 1.5). The α-

chains reside in the plasma membrane such that the immunoglobulin-like domains, which contain the Fc binding site, are extracellular. Except for FcγRII and its isoforms, the FcR α-chains invariably lack signalling motifs in their cytoplasmic tails and must associate with adaptor molecules (typically FcεRIγ) in order to have signalling potential (Ravetch and Kinet, 1991). Classically, cross linking of receptors upon binding to aggregated antibody (typically caused by multivalent antigen) causes phosphorylation of these motifs, recruiting SH2-containing signalling molecules, which, in sufficient numbers, initiates a signalling cascade with a variety of consequences.

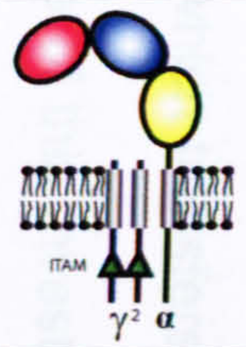
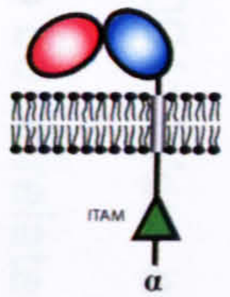
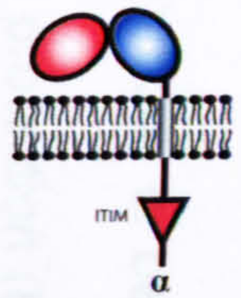
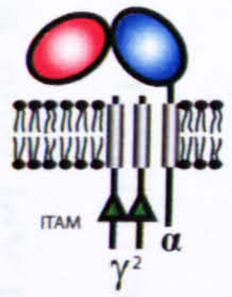
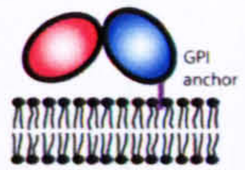
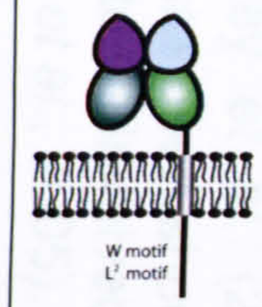
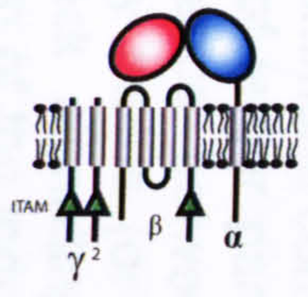
	FcγRI CD64	FcγRIIa CD32a	FcγRIIb CD32b	FcγRIIIa CD16a	FcγRIIIb CD16b	FcRn		FcεRI	FcεRII CD23
									
Protein Family	FcR / Ig	FcR / Ig	FcR / Ig	FcR / Ig	FcR / Ig	MHC / Ig		FcR / Ig	C-type lectin
Cell type	Macrophages Monocytes Neutrophils[l] Eosinophils[l] Dendritic cells	Macrophages Monocytes Eosinophils Neutrophils Platelets Dendritic cells	B cells Macrophages Monocytes Eosinophils Mast cells Dendritic cells	Macrophages Monocytes[l] NK cells Mast cells Dendritic cells	Neutrophils + Soluble form	Placental epithelium Neutrophils Endothelial cells + Soluble form		Mast cells Basophils Eosinophils[l] Dendritic cells Airway smooth muscle *	B cells Monocytes Dendritic cells Airway smooth muscle + Soluble form
Affinity (K_A)	10^8-10^9 M^{-1}	$2x10^6$ M^{-1}	$2x10^6$ M^{-1}	$5x10^5$ M^{-1}	$5x10^5$ M^{-1}	$5x10^7$ M^{-1} at pH 6		10^{10} M^{-1}	10^7 M^{-1}
Specificity	IgG (1=3>4>>2)	IgG (1=3>>2,4)	IgG (1=3>>2,4)	IgG (1,3>>2,4)	IgG (1,3>>2,4)	IgG (1>3>2>4)		IgE	IgE
Function / Effect of ligation	Antigen presentation Inflammation Phagocytosis ADCC Superoxide production (respiratory burst)	Antigen presentation Inflammation Phagocytosis ADCC Superoxide production (respiratory burst)	Down regulation of activation competitive inhibition	Antigen presentation Inflammation Phagocytosis ADCC	Enhanced FcγRIIa activation Regulation by: competitive inhibition CR binding	Placental transcytosis Enhanced phagocytosis Regulation of IgG levels		Release of inflammatory molecules Immediate hypersensitivity Facilitated antigen presentation ADCC	Regulation of IgE levels Facilitated antigen presentation Phagocytosis

Table 1.5 (previous page)
Structural and functional heterogeneity of Fc receptors for human IgG and IgE

Characteristics and expression patterns of human FcγRs and FcεRs. FcγRs have different affinities for the four subclasses of IgG, the values shown are for the subclass with highest affinity. The affinity of the FcγRII & III isoforms is low enough that monomeric IgG dissociates too rapidly to be detectable in cell binding assays. These receptors recognise IgG that is already cross-linked, either in immune complexes (networks of antibodies bound to antigen) or on the surface of larger particles or opsonised bacteria, which have a higher avidity. The balance between the proportion of cross-linked receptors that have activatory motifs (or are associated with activatory subunits) and those that either lack signalling potential or have inhibitory motifs is crucial to the regulation of the induced effector response. Data shown are from Ravetch and Kinet (1991), Cohen-Solal *et al.* (2004), Gould *et al.* (2003), Janeway (2001), McCloskey *et al.* (2007) and S.N. Karagiannis (personal communication). *FcεRI has been shown to be expressed on airway smooth muscle cells and is thought to be involved in triggering airway constriction, particularly in asthmatics (Gounni *et al.*, 2005). CR = complement receptors. I = inducible.

Although a receptor that mediates transcytosis of secreted IgA in mucosal immunity has been characterised in the chicken (Wieland *et al.*, 2004), so far the only IgY-Fc receptor to be cloned is the yolk sac receptor, which allows antibody to be transported into the chicken egg yolk in order to provide neonatal protection (Linden and Roth. 1978; Tressler and Roth, 1987; Morrison *et al.*, 2001; West *et al.*, 2004). Importantly, the yolk sac receptor has been shown to be unrelated to its functional equivalent in mammals, the MHC-related FcRn, which transfers IgG from mother to foetus (Ghetie and Ward, 2000). At the time of this investigation, no leukocyte Fc receptor analogous to receptors for IgG or IgE have been isolated. Previous studies have shown indirect evidence for the existence of such receptors, particularly those mediating phagocytosis of opsonised bacteria (Kogut *et al.*, 2001; Qureshi *et al.*, 1990 & 2000) and hypersensitivity (Faith and Clem, 1973).

1.5.1 Fc receptors that do not bind Fc?

The Fc receptor family is one of many Ig-like immunoreceptor families which all share a common ancestry. Through global genome and EST studies, it is becoming increasingly apparent that Ig-containing receptors have undergone considerable duplication and divergence throughout the jawed vertebrates. Many families have been differentially favoured and expanded in a lineage-restricted manner, so that a great deal of heterogeneity exists between the number of homologues of each family.

A large array of human and mouse FcR family members was recently discovered on the basis of sequence similarity. Initially, these genes were given a host of different designations depending on how they were discovered (Davis *et al.*, 2001; Hatzivassiliou *et al.*, 2001; Mechetina *et al.*, 2002), but for clarity they are now termed "Fc receptor-like" (FCRL or Fcrl) (Maltais *et al.*, 2006). Despite conservation of many of the key residues involved in the binding sites of FcγRI-III and FcεRI, the FCRLs appear to lack the ability to bind Fc (Davis *et al.*, 2002). As yet, no ligands have been found for any FCRL, but several features are consistent with an immunoregulatory role; FCRL appear to be preferentially expressed on B cells (Davis *et al.*, 2001; Hatzivassiliou *et al.*, 2001; Miller *et al.*, 2002) and contain signalling motifs in their cytoplasmic regions that have been shown to be functional in an activating FCRL (Leu *et al.*, 2005) and an inhibitory FCRL (Ehrhardt *et al.*, 2003). This is further supported by links between an FCRL promoter polymorphism and autoimmune disease (Ikari *et al.*, 2006; Umemura *et al.*, 2006), which occurs when immune regulation is dysfunctional.

Closely related sequences to the FcR and FCRL proteins have been noted in several other species but with a wide variation in frequency. In frog, there are more than 70 homologues, termed XFL (Guselnikov, 2004), whereas in fish there may be very few (Stafford *et al.*, 2006). Davis *et al.* suggest that the presence of FcRH genes throughout the jawed vertebrates is an indication of a crucial function that has been conserved (Davis *et al.*, 2005), however it may be the case that convergent evolution has selected different receptor families to perform similar tasks in different species. At the commencement of this investigation, no Fc receptor family members (either FcR-like or FCRL-like proteins) had been reported in the chicken.

1.6 Aims of the investigation

Although chickens were the model for many early experiments in immunology, very little is known about the structure and function of one of their key immunological players, the principal serum antibody, IgY. As recent studies have suggested that this isotype represents a conserved form of the last common ancestor of IgG and IgE, two of the most important mammalian isotypes, IgY is worthy of further investigation. The overall aim of the work reported in this thesis was to initiate a structural and functional investigation into IgY and its Fc receptors. Specific questions to be addressed were:

1) What structural and functional features of IgG and IgE in mammals are conserved from IgY?

There are several similarities and differences in the minimal structural requirements in IgG and IgE for Fc receptor recognition. Several factors were selected for investigation in IgY: presence of the Fab region, presence of Cυ2,

and position of glycosylation and inter-heavy chain disulfide bonds in the Fc region.

2) Which immune cell types in the chicken possess IgY-Fc receptors and what are their structural and functional characteristics?

At the outset of this investigation, no IgY receptors analogous to FcγRI-III or FcεRI-II were reported. In order to begin a study of IgY-Fc receptor binding, cells bearing receptor and/or soluble receptor were required.

3) Following identification of an IgY-Fc receptor, which parts of the IgY structure influence the kinetics of receptor binding?

Given the unusual structure of IgE (Cε2 domains and bent IgE-Fc) and its influence on receptor binding kinetics, the similarity in secondary structure between IgY and IgE poses an interesting question; is the 3D structure of IgY similar to that of IgE and if so, how is IgY able to function as an IgG-like antibody? The IgE:FcεRI interaction is a potential target of allergy therapeutics. A means of reducing the binding affinity or increasing the dissociation rate of IgE from effector cells (i.e. conferring IgG-like kinetics) may be an effective way to prevent long-term sensitisation. Studying how the structure and function of IgE has evolved since the last common ancestor with IgG in order to enhance its anaphylactic properties, may provide some clues as to which structural elements may be the best targets for inhibitory small molecules.

Chapter 2

Materials and Methods

2.1 Materials and stock solutions

The following listing details stock solutions and other materials used in this investigation. All other chemicals are listed with their suppliers in subsequent protocols. Solutions were made up in Milli-Q water unless specified otherwise.

AC elution buffer: 0.2M glycine + 0.1% sodium azide. pH 2.5. 0.45μm filtered, degassed, and stored at 4°C.

AC equilibration / wash buffer: 10mM Tris-HCl + 0.1M sodium chloride + 0.1% sodium azide. pH 8.6. 0.45μm filtered, degassed, and stored at 4°C.

AC neutralisation buffer: 2M Tris-HCl + 0.1% sodium azide. pH 8.6. 0.45μm filtered, degassed, and stored at 4°C.

Acrylamide: 30% (w/v) Acrylamide / Bisacrylamide (Severn Biotech, 20-2100-05) .

Activation buffer (for papain): 100mM Tris-HCl, pH 7.2 + 2mM EDTA + 10mM Cysteine-HCl (Perbio/Pierce, 44889)

Ammonium persulfate (APS): 10 % (w/v) solution stored at 4°C (Sigma, A3678).

Antibodies, see section 2.1.1.

Antibiotics

Ampicillin (1000X): 100mg/ml (Sigma, AG518).

Kanamycin (1000X): 50mg/ml (Sigma, K4000).

Geneticin (G418): 50mg/ml (Gibco, 10131-027).

Hygromycin B: 50mg/ml (Invitrogen, 10687-010).

Blood, chicken: peripheral blood from 4-8 week old broilers, Leonard Ames Ltd., Ampthill, Bedfordshire, UK.

cDNA clones: IMAGE clones obtained from Geneservice:

human FcεRIα in pDNR-LIB (IMAGE 4294467).

human FcRγ in pBS SK- (IMAGE 6129089).

cDNA library, chicken splenocyte: A kind gift from J. Young, BBSRC Institute for Animal Health, Compton, UK.

Cells, see section 2.1.2.

Chloroform/IAA: 24:1 chloroform to isoamyl alcohol, stored at 4°C in the dark.

Competent E.coli:

XL-1 Blue: Stratagene, 200249.

XL-10 Gold: Stratagene, 200314.

TOP 10: Invitrogen, C4040-03.

Coomassie blue stain: Milli-Q water, methanol and acetic acid, 5:5:1 + 0.05% (w/v) Coomassie brilliant blue R250 (Electran, 44418).

Coomassie destain: 5 % methanol + 7.5 % acetic acid.

DEAE sepharose: HiTrap DEAE FF, 5ml prepacked column (GE Healthcare, 17-5154-01).

DLS buffer: 20mM Tris-HCl, pH 7.5 + 25mM sodium chloride.

DNA breaking buffer (vacuum blotting): 0.25M hydrochloric acid.

DNA denaturing buffer (vaccum blotting): 1.5M sodium chloride + 0.5M sodium hydroxide.

DNA gel loading buffer (x6): Promega, G1881.

DNA molecular weight markers

1kb ladder: Promega, G6941.

100bp ladder: Promega, G8291.

λ / EcoRI / HindIII: Prepared in house (P. Marsh). 300μg/ml in 10mM Tris-HCl, pH 8.0 + 20mM sodium chloride + 10mM EDTA, 5% (v/v) glycerol + 0.025% (w/v) Bromophenol Blue.

DNA neutralisation buffer (vacuum blotting): 1.5M sodium chloride + 0.5M Tris-HCl, pH 7.0.

DNA transfer buffer (vacuum blotting): 1.5 M sodium chloride + 100mM sodium hydrogen phosphate + 10mM EDTA. pH 7.4.

DTT: 1M Dithiothreitol (Sigma, D9779), prepared fresh before use.

EDTA: 0.5M disodium ethylenediamine tetraacetic acid, pH 8.0. Sodium hydroxide was added until EDTA was dissolved, then pH adjusted to pH8.0 with concentrated hydrochloric acid.

ELISA Coating buffer:15 mM Sodium carbonate + 35 mM Sodium hydrogen carbonate. pH 9.6.

ELISA phosphate-citrate buffer: 0.05 M phosphate-citrate buffer + 0.03% sodium perborate (Sigma, P4922).

Ethidium bromide: 10mg/ml (Sigma, E1510).

Formaldehyde (37%): Sigma, F1635.

Freeze medium (N_2 storage of cells): 10% DMSO (Sigma, D2650) + 90% FBS.

G/A (100X): 600mg Glutamate (Sigma, G-5638) + 600mg Asparagine (Sigma, A-4159) in 100ml Milli-Q water. 0.2μm filtered and stored at -20°C

HPLC running buffer: 0.5M Tris-HCl + 0.25M sodium chloride + 0.05% (w/v) sodium azide. pH 7.2. Degassed and 0.2μm filtered.

IE bind / wash buffer: 20mM Tris-HCl + 25mM sodium chloride. pH 7.5.

IE elution buffer: 20mM Tris-HCl + 2M sodium chloride. pH 7.5.

Luria-Bertani (LB) agar: 32g/L in Milli-Q water, autoclaved at 121°C for 15 minutes (Invitrogen, 22700-025).

Luria-Bertani (LB) broth: 20g/L in Milli-Q water, autoclaved at 121°C for 15 minutes (Invitrogen, 1278-052).

Lysis buffer (for cell membrane protein solubilisation): 0.5% NP-40 (BDH, 56009) or Triton X-100 (Sigma, T8787) + 10mM Tris, pH 7.5 + 25mM potassium chloride + 120mM sodium chloride + 1X Complete protease inhibitors (Roche, 1873580).

Media (cell culture), see section 2.1.2.

Nucleosides (50X): 35mg Adenosine (Sigma, A-4036) + 35mg Guanosine (Sigma, G-6264) + 35mg Cytidine (Sigma, C4654) + 35mg Uridine (Sigma, U-3003) + 12mg Thymidine (Sigma, T-1895) in 100ml Milli-Q water. 0.2μm filtered and stored at -20°C.

Nitrocellulose blotting membrane: Protran BA83 0.2μm (Schleicher and Schuell, 10401396).

Oligonucleotides / Primers, see section 2.1.4.

Papain digestion buffer: 0.1M sodium phosphate buffer, pH 7.0 (see below) + 3mM EDTA + 10mM L-cysteine (Sigma, W326305).

PBS (Phosphate buffered saline, 10X): 1.37M Sodium chloride + 27mM Potassium chloride + 80mM Disodium hydrogen phosphate + 15mM Potassium dihydrogen phosphate. pH 7.4. 0.05% sodium azide added to 1X PBS when used as a protein storage buffer.

PBS (for cell methods, 1X): D-PBS, Invitrogen, 14190-094.

PBS-Marvel: 1X PBS + 2% (w/v) Marvel powdered milk (Premier international foods).

PBS-Tween: 1X PBS + 0.05% (v/v) Tween-20 (Sigma, P5927)

PBS-Tween-Marvel: 1X PBS + 0.05% (v/v) Tween-20 (Sigma, P5927) + 2% (w/v) Marvel powdered milk (Premier international foods).

PBS-BSA: 1X PBS + 1% (w/v) bovine serum albumin (Sigma, A7030) + 0.05% sodium azide.

Phenol: Chloroform: isoamyl alchohol (25:24:1): Sigma, P-2069.

Ponceau Red stain: 1.25g (0.25% w/v) Ponceau S + 200ml methanol + 75ml acetic acid + 225ml Milli-Q water.

Plasmids, see section 2.1.3:

Protein molecular weight standards:

For SDS-PAGE: SeeBlue Plus 2 (Invitrogen, LC5925).

For size exclusion chromatography: Gel filtration molecular weight marker kit (Sigma, MW-GF-1000).

PVDF membrane: Sequi-Blot PVDF (Biorad, 162-0186).

SDS stock for PAGE gels: 10% (w/v) sodium dodecyl (Lauryl) sulphate (Sigma, L3771).

SDS-PAGE loading buffer (4X): 8% SDS + 40% Glycerol + 20% β-mercaptoethanol + 0.008% bromophenol blue + 0.25M Tris-HCl. pH 6.8.

SDS-PAGE running buffer (10X): 0.25M Tris + 1.92M Glycine + 1% SDS. pH 8.3.

Semi-dry transfer anode buffer: 0.4M Hexanoic acid (ε-amino-n-caproic acid) + 0.025M Tris + 20% (v/v) methanol. pH 9.4.

Semi-dry transfer cathode buffer: 0.3M Tris + 20% (v/v) methanol. pH 10.4.

SOC medium: Invitrogen, 15544-034.

Sodium phosphate buffer (pH 7.0): 19.5ml 0.2M monobasic sodium phosphate + 30.5ml 0.2M dibasic sodium phosphate. pH 7.0.

Southern hybridisation buffer: ULTRAhyb (Ambion, 8670).

Standard saline citrate (SSC, 20X): 3M sodium chloride + 0.3M sodium citrate. pH 7.0.

Streptavidin-RPE: Dako cytomation, R0438.

Stock isotonic Percoll: 90% Percoll, 1,130 g/ml (Amersham, 17-0891-01) + 10% 1.5M sodium chloride.

Superdex 200: Pharmacia/GE Heathcare, 17-5175-01.

TBE (10X): 0.89M Tris + 0.89M Boric acid + 0.02M EDTA. pH 8.3.

TBS (10X): 1M Tris-HCl + 1.5M Sodium chloride. pH 7.5. 0.05% sodium azide added to 1X TBS when used as a protein storage buffer.

TE: 10mM Tris-HCl, pH7.6 + 1.0mM EDTA, pH 8.0.

TEMED: N, N, N', N'-tetramethylethylenediamine (Sigma, T-9281).

Trypan blue solution (0.4%): Sigma, T8154.

2.1.1 Antibodies, fragments and conjugates

Species	Polyclonal / Monoclonal	Reactivity	Conjugate	Supplier	Catalogue number	Other info
Goat	P	IgY-Fc	-	ICL	GGFC-30A	*
Rabbit	P	IgY	HRP	Promega	G135A	
Rabbit	P	IgY	FITC	Promega	G269A	
Donkey	P	IgY	-	Chemicon	AP194	
Donkey	P	IgY	Agarose beads	Gallus Immunotech	DAIgY-AGA-5	
Mouse	M	IgY	-	Sigma	C7295	clone CG-106†
Rabbit	P	Goat Igs	FITC	Abcam	ab6737-1	
Mouse	M	FLAG tag	-	Sigma	F1804	
Mouse	M	V5 tag	-	Sigma	V-8012	clone V5-10
Goat	P	Mouse Igs	FITC	Dako	F0479	$F(ab')_2$
Rabbit	P	Human IgGs	FITC	Dako	F0315	$F(ab')_2$
Mouse	M	human FcεRIα	-			clone 15-1‡

Table 2.1.1 i
Primary and secondary antibodies used in immunoassays
* Immunogen was papain-derived IgY-Fc.
† Antibody was found to react with Cυ2-4 but not Cυ3-4 (data not shown), therefore epitope is likely to be in Cυ2.
‡ Antibody prepared in house (S. Karagiannis), cells originally a gift from J.-P. Kinet (Wang *et al.*, 1992).

Species	Fragment	Supplier	Catalogue number	Other info
Human	IgE-Fc (Cε2-4)			N265Q/N371Q Made in house (Young *et al.*, 1995)
Human	IgG1	Sigma	I-5154	
Human	IgG1-Fc	Bethyl Laboratories	P80-104	
Rabbit	IgG	Sigma	I-15006	
Chicken	IgY (Serum)	Jackson Immunoresearch	003-000-003	
Chicken	IgY (Yolk)			Prepared in house from egg yolk
Chicken	IgY-$(Fab)_2$	Rockland	003-0104	Sold as $F(ab)_2$, but found to be single Fab (data not shown)

Table 2.1.1 ii
Antibodies and antibody fragments used as test material

2.1.2 Cell lines & media

MQ-NCSU: chicken monocyte-like cells, a kind gift from M.A. Qureshi (Qureshi *et al.*, 1990) , North Carolina State University, Raleigh, USA.

38.5% Lebovitz L-15 medium (Invitrogen 11415-056)

38.5% McCoy's 5a medium (Invitrogen, 22330-070)

5% tryptose phosphate broth (Invitrogen, 18050-039)

8% ultra-low IgG foetal bovine serum (Invitrogen, 16250-078)*

2mM L-glutamine (Invitrogen, 25030-024)

11 µg/ml sodium pyruvate (Invitrogen, 11360-039)

10µM 2-mercaptoethanol (Invitrogen, 31350-010)

1% penicillin/streptomycin (Invitrogen, 15140-122)

1% amphotericin B desoxycholate ('Fungizone', Invitrogen, 15290-026)

* Original MQ-NCSU culture media ('LM-Hahn') contains 10% chick serum (Sigma, 5405) in addition to 8% FBS (Invitrogen, 10270-106); the medium was modified so that IgY would be absent.

CHO-K1: Chinese hamster ovary cell line (Kao and Puck, 1968; Puck *et al.*, 1958). ECACC No. 85051005.

D-MEM (Sigma, D6046)

10% FBS (Invitrogen, 10270-106)

1% penicillin/streptomycin/glutamine (Invitrogen, 10378-016).

NS-0: Mouse myeloma cell line that is phenotypically glutamine synthetase (GS) negative, so can be stably transfected with GS vectors (Bebbington *et al.*, 1992; Galfre and Milstein, 1981; Young *et al.*, 1995). ECACC No. 85110503.

Non-selective NS-0 media:

IMDM w/o L-glutamine (Sigma, I3390)

2mM Glutamine (Invitrogen, 25030-024)

10% FBS (Invitrogen, 10270-106)

1 % penicillin/streptomycin (Invitrogen, 15140-122)

Selective NS-0 media (Glutamine free):

IMDM w/o L-glutamine (Sigma, I3390)

10% Dialysed FBS (Invitrogen, 26400-044)

1X Nucleosides (see section 1.1)

1X G/A (see section 1.1)

1X Non-essential amino acids (Invitrogen, 11140-035)

1 % penicillin/streptomycin (Invitrogen, 15140-122).

HEK 293: Human embryonic kidney cell line (Graham *et al.*, 1977). ATCC No. CRL-1573. Same growth medium as CHO-K1 above.

U937: Human monocyte-like cell line (Sundstrom and Nilsson, 1976). Cells were a kind gift from J.-P. Kinet, Harvard University, Cambridge, USA.

RPMI 1640 (Invitrogen, 31870-025)

10% FBS (Invitrogen, 10270-106)

1% penicillin/streptomycin/glutamine (Invitrogen, 10378-016).

RBL-SX38: Rat basophilic leukaemia cell line transfected with human FcεRI subunits α, β, and γ (Wiegand *et al.*, 1996). Cells were a kind gift from J.-P. Kinet, Harvard University, Cambridge, USA.

MEM (Invitrogen, 31095-029)

10% FBS (Invitrogen, 10270-106)

1% penicillin/streptomycin/glutamine (Invitrogen, 10378-016).

2.1.3 Plasmid vectors

pRY24: Mammalian expression vector based on pMRR018 / pEE6hCMV (Stephens and Cockett, 1989), modified to encode human IgE-Fc Cε2-4 (Young *et al.*, 1995). Contains a gene encoding glutamine synthetase. Selected in *E.coli* using ampicillin resistance.

pSP64T: Cloning vector (Genbank accession no. X65327), previously commercially available (Promega). Vector was a kind gift from P. Marsh, King's College London. Selected in *E.coli* using ampicillin resistance.

pCDNA3: Mammalian expression vector containing neomycin/G418 resistance gene (Invitrogen, V79020). Selected in *E.coli* using ampicillin resistance.

pCDNA3.1/Hygro: Mammalian expression vector containing hygromycin resistance gene (Invitrogen, V87020). Selected in *E.coli* using ampicillin resistance.

pCR4-TOPO: TOPO vector used to clone PCR products with T/A ends for DNA sequencing (Invitrogen, 45-0071). Selected in *E.coli* using kanamycin resistance.

2.1.4 Oligonucleotides

All oligonucleotides were synthesised by MWG Biotech.

Oligonucleotide	Notes
5' - TAAATCCCGGTAAATGAGTTTAAACCTGCCTCCCTCCCTCCCAGG - 3' 5' - CTGGGAGGGAGGGAGGCAGGTTTAAACTCATTTACCGGGATTTAC - 3'	Mutagenesis primers to introduce PmeI restriction site into pRY24
5' - TGCTCAAAAAGGAGGATATCGTCGCCCGAGTAGGGCCCCCACTG - 3'	Forward primer used to amplify Cu2-4 from cDNA library with EcoRV restriction site
5' - GTGGAGGATCACGTCGATATCTGCCCGGACGGCGCTCAG - 3'	Forward primer used to amplify Cu3-4 from cDNA library with EcoRV restriction site
5' - CGAGGGTTTCGCACCCGTTTAAACTTATTTACCAGCCTGTTTCTG - 3'	Reverse primer used to amplify Cu2-4 and Cu3-4 from cDNA library with PmeI restriction site
5' - AGCCTGAGCAGCCGCGTCCAGGTCAGCGGCACCGATTGG - 3' 5' - CCAATCGGTGCCGCTGACCTGGACGCGGCTGCTCAGGC - 3'	Mutagenesis primers to introduce N308Q mutation into IgY-Fc
5' - GTCCTCCAAGAACACTTCCAGGGCACCTACAGCGCCAGC - 3' 5' - GCTGGCGCTGTAGGTGCCCTGGAAGTGTTCTTGGAGGAC - 3'	Mutagenesis primers to introduce N407Q mutation into IgY-Fc
5' - GCCAGATGTGATATCTGCCCGGACGGCGCTCAG - 3' 5' - CTGAGCGCCGTCCGGGCAGATATCACATCTGGC - 3'	Mutagenesis primers to introduce C340S mutation into IgY-Fc
5' - GACGGCGCTCAGAGCTGCAGCCCCATCCAGCTG - 3' 5' - CAGCTGGATGGGGCTGCAGCTCTGAGCGCCGTC - 3'	Mutagenesis primers to introduce C347S mutation into IgY-Fc
5' - ATGTGATATCTGCCCGGACGGCGCTCAGAGCTGCAGCCCCATCC - 3' 5' - GGATGGGGCTGCAGCTCTGAGCGCCGTCCGGGCAGATATCACAT - 3'	Mutagenesis primers to introduce C340S and C347S mutations into IgY-Fc
5' - TGGCAAACTCCTGGAGCACACAGAAAC - 3'	chFcR/L EC1 forward 'gene specifc primer' for RACE PCR
5' - GGCATTCTGGATCCTGTACGTGTTGGTT - 3'	chFcR/L EC1 reverse 'gene specifc primer' for RACE PCR
5' - GCAGTTTGGCTGCGGTGCGGTTCTAC - 3'	chFcR/L EC2 forward 'gene specifc primer' for RACE PCR
5' - GTTTGGTGATGTCGGCTCCATCCCTG - 3'	chFcR/L EC2 reverse 'gene specifc primer' for RACE PCR
5' - TCCTCTGAGTACCAGGTGCCAGCAGTGG - 3'	chFcR/L EC3 forward 'gene specifc primer' for RACE PCR
5' - CAGGAGTAGAACCCCGTGTCCATCAGCC - 3'	chFcR/L EC3 reverse 'gene specifc primer' for RACE PCR
5' - TCGACCCAACAGAGTGACAACGGGC - 3'	chFcR/L EC4 forward 'gene specifc primer' for RACE PCR
5' - ACCTTCAGGCTCCGGGCTGGGCT - 3'	chFcR/L EC4 reverse 'gene specifc primer' for RACE PCR
5' - CTAGACAGGTGGTTCGGGATG - 3'	chFcR/L forward primer to obtain 'full-length' CDS
5' - TGGGTTTACGGCGCTCCT - 3'	chFcR/L reverse primer to obtain 'full-length' CDS
5' - ACTGGCAGAGCCGGAGCTGTGCTACG - 3'	chFcRg forward primer to obtain 'full-length' CDS
5' - TGGTGGTGGGGGGGTCCGAACTCAGG - 3'	chFcRg reverse primer to obtain 'full-length' CDS
5' - GATGGCGGTGGATATCGGTAAGCCTATCCTAACCCTCTCCTC... ...GGTCTCGATTCTACGGAGATGGCCCTGCTGACC - 3'	Forward primer used to amplify chFcR/L CDS with EcoRV restriction site and V5 tag
5' - GTGGGTTTACGTTTAAACTCAAACATCCCCT - 3'	Reverse primer used to amplify chFcR/L CDS with PmeI restriction site

Oligonucleotide	Notes
5' - CGCTCTGCTGGATATCGACTATAAGGACGATGATGAC... ...AAGGAGCCGGAGCTGTGC - 3'	Forward primer used to amplify chFcRg CDS with EcoRV restriction site and FLAG tag
5' - GGTGGTGGGGGGGTTTAAACTCAGGCTTTGTGC - 3'	Reverse primer used to amplify chFcRg CDS with PmeI restriction site
5- GGGCTGCAGGTCGATCG -3'	Forward sequencing primer complimentary to B72.3 VLk leader
5- CGGAGGTGCAGGTCCTC -3'	Forward sequencing primer complimentary to Cu2
5- GGACGGCGCTCAGAGC -3'	Forward sequencing primer complimentary to Cu3
5- CGTATTCCTGTAGACGCTC -3'	Reverse sequencing primer complimentary to Cu3
5- TTATTTACCAGCCTGTTTC -3'	Reverse sequencing primer complimentary to Cu4
5- CTCTGAGCAGGCACAG -3'	Reverse sequencing primer complimentary to hulgE UTR
5- ATGTATCATACACATACGAT -3'	Forward sequencing primer for pSP64T
5- CAAAGTTGAGCGTTTATTCTGAG -3'	Reverse sequencing primer for pSP64T

Table 2.1.4 (above and previous page)
Oligonucleotide primers used for cDNA amplification, plasmid mutagenesis, nucleotide sequencing and bacterial colony screening

2.2 Nucleic acid methods

Agarose gel electrophoresis (analytical)

Plasmids, RNA, PCR products and DNA digests were analysed using gels composed of 1-2% agarose (Sigma, A9539) in TBE with 0.5g/ml ethidium bromide. Boiled agarose solution was poured and set in a gel tray with a gel comb, before being placed into a gel tank and submerged in TBE with 0.5g/ml ethidium bromide. Samples were diluted in DNA gel loading buffer and loaded adjacent to appropriate DNA molecular weight markers. Gels were run at 100V until sufficient separation of loading dye was seen. DNA/RNA was visualised by UV transillumination (320nm) using a Gene Genius bio-imaging system (Syngene).

Agarose gel electrophoresis (preparative)

Restriction digestion products were separated using agarose gel electrophoresis as described above, except that gels were made with SeaKem GTG agarose (Cambrex, 50071). Bands representing the desired fragments were visualised by UV transillumination, excised using a sterile razor blade and electro-eluted into 200µl TBE in a dialysis bag (Medicell, DTV12000.02.000).

5'-dephosphorylation of linearised plasmids

5' phosphate groups were removed from linearised plasmid vector DNA in order to reduce self-ligation in ligation reactions. Shrimp alkaline phosphatase (Promega, M8201), 1 Unit/µg DNA, was mixed with each sample in dephosphorylation buffer (10X stock supplied with enzyme: 0.5M Tris-HCl, pH 9.0 + 100mM Magnesium chloride) in a total reaction volume of 30µl. Samples were heated at 37°C for 15 minutes, then at 65°C for a further 15 minutes, in order to heat inactivate the enzyme. Enzyme and buffer were removed using phenol:chloroform extraction and ethanol precipitation.

Estimation of nucleic acid concentration

DNA and RNA concentration were calculated from the absorbance of solutions at 260nm (see 'Estimation of protein concentration' for explanation), measured using a Cary 50 spectrophotometer (Varian):

Concentration of **DNA** (µg/ml) = A_{260} x (dilution factor) x 50 µg **DNA**/ml/A_{260} unit

Concentration of **RNA** (µg/ml) = A_{260} x (dilution factor) x 40 µg **RNA**/ml/A_{260} unit.

Ethanol precipitation of DNA

Samples were mixed with 1/10th volume 3M sodium acetate, pH 5.2, then 2 volumes of ice-cold 100% ethanol and cooled at -20°C for 1 hour in order to precipitate DNA. Samples were centrifuged at 13,000 rpm at 4°C for 30 minutes and the supernatant discarded. Pellets of DNA were washed with 70% ethanol and spun for a further 10 minutes. Supernatant was removed and pellets dried using a vacuum centrifuge. Dried pellets were resuspended to the desired concentration in either autoclaved water or TE.

Propagation and storage of transformed bacterial colonies

Individual bacterial colonies resulting from selection on LB-agar with antibiotic were picked using a sterile pipette tip and transferred to sterile LB-broth containing the same antibiotic. Cultures were grown at 37°C for ~16 hours with shaking (220rpm). A culture volume appropriate to the required quantity of plasmid DNA to be extracted was prepared (see 'Purification of plasmid DNA from bacteria'). For cultures exceeding 100ml, a 5ml starter culture was grown for ~8 hours at 37 °C with shaking and used to inoculate the main culture (1:250). For storage, a 1.5ml aliquot of 16 hour culture was removed and vortexed with 0.5ml sterile 60% glycerol. Glycerol stocks were snap frozen in liquid nitrogen and stored at -70°C.

Ligation of DNA fragments

Gel purified DNA inserts prepared by restriction digestion were ligated into dephosphorylated, linearised plasmids using T4 DNA ligase (Promega, M1801). Generally, a 3:1 molar ratio of insert:vector was used with 100ng

vector and 1 unit of enzyme in ligase buffer (10X stock supplied with enzyme: 300mM Tris-HCl, pH 7.8 + 100mM Magnesium chloride + 100mM DTT + 10mM ATP), with a final reaction volume of 10μl. For sticky-end ligation (5' or 3' overhangs), the reaction was performed at room temperature for 3 hours. For blunt end ligation, the reaction was performed at 15°C overnight.

Polymerase chain reaction (PCR) methods

PCR using the primers specified in section 2.1.4 was used for several applications:

(1) Bacterial colony screening. The presence of plasmid was assayed following transformation with ligation reaction products and selection on LB-agar with appropriate antibiotic. A sterile pipette tip was used to pick each colony and resuspend it in 20μl sterile water. 10μl was removed, boiled for 5 minutes and spun at 13,000 rpm in a microfuge. 1μl supernatant was used in a PCR reaction. For this application, fidelity of replication was not important, so a pre-mixed *Taq* polymerase cocktail was used, PCR Master Mix (Promega, M7502).

(2) Rapid amplification of cDNA ends (RACE-PCR). A SMART RACE kit (BD Biosciences/Clontech, 634914) was used to amplify previously unknown 5' and 3' ends of chFcR/L cDNA fragments, following the manufacturer's protocol. Briefly, cDNA was reverse transcribed from MQ-NCSU-derived RNA, with known sequences incorporated at the 5' and 3' ends. Primers complimentary to these known sequences, together with 'gene specific' primers (see section 2.1.4) were used to amplify cDNA fragments, which

were then isolated using agarose gel electrophoresis, T/A cloned and sequenced.

(3) Amplification of 'complete' cDNA. IgY-Fc cDNA was amplified from a chicken splenocyte cDNA library and both chFcR/L cDNA and chFcRγ cDNA were amplified from reverse transcribed RNA purified from MQ-NCSU cells or primary chicken basophils. For preparation of a Southern hybridisation probe, forward and reverse sequencing primers specific for exon III (encoding domain EC3 of chFcR/L) were used.

(4) Mutagenesis of plasmid vectors. Site-directed mutagenesis was performed using a Quikchange II XL kit (Stratagene, 200521-5), as per the manufacturer's instructions, using primers specified in section 2.1.4. Briefly, PCR using *Pfu* proofreading polymerase was carried out in which mutagenic primers were annealed to the plasmid to be altered and extended in order to synthesise new strands incorporating the desired mutation. After 18 cycles, the original unmodified parental strands were digested using a *dam*-methylation-sensitive restriction enzyme. Mutated plasmids were transformed into XL-10 gold competent *E.coli*, in order to repair nicks and replicate the plasmid. Sufficient plasmid DNA was extracted to allow the nucleotide sequence to be determined to confirm the presence of the mutation.

In most instances, a typical PCR reaction was prepared on ice as follows, using a high-fidelity proofreading polymerase:

5μl 10X **PCR buffer** for *Pfu* (Supplied with *Pfu*: 200mM Tris-HCl, pH 8.8 + 100mM potassium chloride + 100mM ammonium sulfate + 20mM magnesium sulfate + 1% Triton X-100 + 1mg/ml BSA)

1µl 50X **dNTP mix**, 10mM each dNTP (Promega, 1511)

1µM **Forward primer** (see section 2.1.4)

1µM **Reverse primer** (see section 2.1.4)

0.5µg **Template plasmid**, or 2.5µg cDNA library

1.25 Units ***Pfu* polymerase** (Promega, M774A)

Final volume 50µl with sterile water.

Most reactions were run with the following profile in a GeneAmp 9700 PCR system (Applied Biosystems):

95°C 2 minutes (Initial Denaturation)

~25 cycles {
- 95 °C 1 minute (Denaturation)
- X°C 30 seconds (Annealing)
- 74 °C 2 minutes per kb of amplicon (Extension)

74 °C 5 minutes (Final extension)

4 °C indefinite (Soak/End)

The annealing temperature (marked X) was determined by the primer pair used, and usually had to be optimised. The melting temperature of each primer was calculated using MWG Biotech's primer design tool and roughly 5°C above the highest melting temperature was used as the annealing temperature.

Purification of plasmid DNA from bacteria

Plasmid DNA was extracted from bacterial clones grown overnight in selective LB-broth using a Qiagen purification kit, according to the manufacturer's protocol. The quantity of culture volume and the kit used depended on the required amount of DNA. For cloning methods and sequencing, up to ~20µg was prepared using a 'QIAprep Spin miniprep' kit

(Qiagen, 27104). For applications where larger quantities were required (e.g. transfection by electroporation), a 'Hispeed Plasmid midiprep' kit (Qiagen, 12643) or a 'QIAfilter Plasmid maxiprep' kit (Qiagen, 12262) was used. For sensitive applications, or if a high concentration of DNA was required, plasmid DNA was further purified using phenol:chloroform extraction and/or ethanol precipitation.

Purification of RNA from cells

Total cell RNA was prepared from MQ-NCSU cells and a crude preparation of chicken peripheral blood basophils using a 'Midas pure RNA' kit (Biogene, BG-SPR/50). The manufacturer's protocol was followed, with the addition of an genomic DNA digestion step, using RNase-free DNase I (Qiagen, 79254). For each column, 30 Kunitz units of DNase I were diluted in the supplied digestion buffer to a final volume of 75µl and incubated with RNA on the column membrane for 15 minutes at room temperature. Purified RNA was eluted in DEPC-treated water, snap frozen in liquid nitrogen and stored at -70°C.

Reverse transcription of RNA

For RACE-PCR (see 'PCR methods'), cDNA was synthesised using reagents and protocols included in the SMART RACE cDNA amplification kit (BD Biosciences/Clontech, 634914). For other applications, a first strand RT reaction was prepared as follows: 1X first-strand buffer (supplied with Superscript II enzyme) + 5mM DTT (Invitrogen, Y00147) + 0.4mM dNTPs (Invitrogen, 18427-013) + 0.2ng oligo dT primer (Promega, C1101) + 0.05ng

random primers (Promega, C1181) + 8 units RNaseOUT RNase inhibitor (Invitrogen, 10777-019) + 1μg RNA, to a final volume of 40μl with nuclease-free water (Promega, P1193). Prior to the addition of 400 units Superscript II reverse transcriptase (Invitrogen, 18064-022), the reaction mixture was heated at 70°C for 2 minutes to denature the RNA, then immediately placed on ice. The reaction was carried out by incubation at 37°C for 10 minutes, 42°C for 45 minutes, and 50°C for 10 minutes. 160μl of nuclease-free water was added to the reaction and the enzyme was inactivated by heating at 100°C for 2 minutes. cDNA was stored at -20°C.

Restriction digestion

Plasmid DNA or PCR products were digested using one or more of the following restriction enzymes: EcoRI (Promega, R6011), EcoRV (Promega, R6351), HindIII (Promega, R6041), PmeI (New England Biolabs, R0560S), SalI (Promega, R6051) or XhoI (Promega, R6161). Generally, reactions were set up as follows; where a double digest was required, the appropriate reaction buffer was chosen from the Promega '4-core' buffer set (Promega, R9921). 2μl 10X restriction buffer (supplied with enzyme or from '4-core' set) + 2μg acetylated BSA (supplied with enzyme)+ 1μg DNA + 5 units restriction enzyme, to a final volume of 20μl using autoclaved Milli-Q water. For a preparative restriction digestion, the reaction was scaled up 5-10-fold and the digestion products separated using agarose gel electrophoresis.

Phenol:chloroform extraction

Phenol:chloroform extraction was used to separate protein contaminants of DNA solutions. An equal volume of phenol:chloroform:isoamyl alcohol, 25:24:1 (Sigma, P-2069), was added to the sample and emulsified using a vortex mixer. The organic and aqueous phases were separated by centrifugation at 13,000 rpm in a microfuge for 1 minute at room temperature. The aqueous phase was removed and extraction repeated until no protein was visible at the interface of the two phases. To remove phenol, an equal volume of chloroform:isoamyl alcohol, 24:1, was added to sample, mixed, spun and the aqueous phase removed as above. Ethanol precipitation was performed to recover the purified DNA.

Radiolabelling of single-stranded cDNA

A single stranded radiolabelled cDNA probe was prepared for Southern hybridisation using DNA labelling beads (GE Heathcare / Amersham, 27-9240-01) with [α-^{32}P]-dCTP (GE Heathcare / Amersham, AA0075). A double-stranded cDNA fragment comprising part of exon III of chFcR/L was amplified by PCR, then purified using phenol:chloroform extraction and ethanol precipitation. DNA at ~100ng/μl in TE was denatured to produce a single-stranded species by heating at 100°C for 2 minutes, then immediately cooling on ice for 2 minutes. The reaction was prepared by adding 50ng DNA to 50μCi [α-^{32}P]-dCTP and a labelling bead. Milli-Q water was added to a final volume of 50μl, and the mixture incubated at 37°C for >15 minutes. The labelled probe was used immediately.

Sequence determination

Nucleotide sequencing was performed by MWG Biotech, or by P. Marsh and R. Chen, King's College London, using the fluorescent DNA dideoxy chain termination method (Sanger *et al.*, 1977). Sequences were analysed and aligned using ChromasPro software v1.3 (Technelysium).

Southern hybridisation (Southern blot)

PCR reactions to amplify 'full length' chFcR/L cDNA from MQ-NCSU or chicken peripheral blood basophils (contaminated with erythrocytes) were suspected to contain numerous products resulting from non-specific priming. To establish which products contained chFcR/L exons, a Southern hybridisation was performed using a radiolabelled probe complimentary to exon III of chFcR/L (see 'Radiolabelling a single-stranded cDNA probe'). PCR reactions were separated using agarose gel electrophoresis and transferred to nitrocellulose membrane (see 'Vacuum transfer of DNA'). The membrane was placed in a hybridisation bottle with ~10ml Southern prehybridisation / hybridisation buffer (Ambion, 8670) for 30 minutes at 42°C in a hybridisation oven. The buffer was removed and replaced with a fresh buffer and 10 µl (~10ng) of newly labelled ^{32}P-ssDNA probe and incubated at 42°C overnight. The membrane was repeatedly washed at 42°C as follows: once with 5X SSC + 0.1% SDS for 5 minutes, twice with 1X SSC + 0.1% SDS for 5 minutes each, once with 0.2X SSC + 0.1% SDS for 15 minutes, and once with 0.1X SSC + 0.1% SDS for 15 minutes. The membrane was covered with a sheet of Saran wrap and used to expose a piece of Fuji RX X-ray film (GRI, JTS010) for 1 hour in an X-ray cassette with intensifying screens. The film was developed using an

X-OMAT developer (Kodak). The membrane was used to expose another piece of film for a further ~16 hours to ensure no further bands were visible.

T/A cloning of PCR products

In order to obtain high-quality sequences of RACE-PCR products, fragments were separated using agarose gel electrophoresis and cloned using a TOPO T/A cloning kit (Invitrogen, 45-0071), following the manufacturer's protocol. The RACE PCR products were amplified using the Advantage II polymerase mix (BD Biosciences, 639207), so contained 3'-adenine overhangs, which could be reacted with linearised pCR4-TOPO vector, containing 3'-thymidine overhangs. Reacted vectors were used to transform TOP10 competent *E.coli*, and positive clones (assayed by PCR) were propagated. Plasmids containing the various RACE PCR products were extracted and sequenced.

Transformation and selection of competent cells

Competent *E.coli* were used to clone and amplify plasmid DNA encoding antibiotic resistance genes. *E.coli* were stored at -70°C, then thawed on ice immediately before transformation. ~10ng pre-chilled DNA was added to 50µl *E.coli* (~10^8 cells), mixed gently by flicking the tube, and incubated for 30 minutes on ice. The cells were then heat shocked at 42°C for 30-90 seconds (depending on the strain of *E.coli* used) and placed on ice for a further 2 minutes. 0.9ml SOC medium (pre-warmed to 37°C) was added and bacteria incubated for 1 hour at 37°C with shaking (220 rpm). Bacteria were plated onto

selective LB-agar (5, 50 and 500µl per petri dish), containing either ampicillin (100µg/ml) or kanamycin (50µg/ml), and grown overnight at 37°C.

Vacuum transfer of DNA

In preparation for Southern blotting, DNA in a 1% agarose gel was vacuum transfered onto nitrocellulose blotting membrane. The gel was soaked in DNA breaking buffer, DNA denaturing buffer and DNA neutralisation buffer for 30 minutes each, rinsing with Milli-Q water between buffers. A piece of blotting membrane was cut to the size of the gel and placed together with plastic sealing sheets, into an LKB 2016 VacuGene vacuum blotting system (Pharmacia). The gel and membrane were covered with DNA transfer buffer and blotted for ~90 minutes. Transferred DNA was crosslinked onto the blotting membrane using a Stratalinker 1800 UV crosslinker (Stratagene) for 60 seconds.

2.3 Protein methods

Acetone precipitation of protein

Acetone precipitation was used to prepare samples for SDS-PAGE analysis when proteins were of insufficient concentration (>0.1 mg/ml) or in a buffer unsuitable for electrophoresis (e.g. high salt). Four volumes of cold acetone (-20°C) was added to the sample, mixed by inversion and incubated at -20°C for 1 hour. The sample was centrifuged at 14,000xg at 4°C and the supernatant discarded. The protein pellet was dried in a vacuum centrifuge and resuspended in the desired buffer (e.g. 1X SDS-PAGE loading buffer).

Affinity chromatography

Affinity chromatography was used to purify recombinant IgY-Fc from culture supernatant of stably transfected NS-0 cells and to extract IgY from solubilised membrane preparations, following incubation of MQ-NCSU cells in chicken serum IgY. In both instances, the affinity matrix consisted of goat polyclonal anti-IgY coupled to agarose beads (Gallus Immunotech, DAIgY-AGA-5). 5ml slurry was packed into a glass 10mm diameter Econo-column (Bio-rad, 737-1011) with an appropriate flow adaptor (Bio-rad, 738-0015). The column was run at 4°C using a peristaltic pump at a flow rate of 0.75ml/min. Before each run, the column was regenerated with 25ml AC elution buffer, then equilibrated with 50ml AC equilibration/wash buffer. For preparative runs, sample (supernatant or solubilised protein preparation) was filtered through a 0.45µm cellulose acetate membrane (Sartorius, 11106—47------N) then re-circulated through the column until saturation of the affinity matrix. The column was washed with 100ml AC equilibration/wash buffer and bound material eluted

using 10ml AC elution buffer. The sample was immediately neutralised using AC neutralisation buffer (~20µl per ml). An automated microfluidics system (constructed in-house by A.J. Beavil) was used to perform multiple load/wash/elute cycles. For analytical runs, a similar protocol was used, except 100µl fractions of eluate were collected, acetone precipitated, resuspended in 1X loading buffer and analysed using SDS-PAGE.

Biotinylation of antibodies and antibody fragments

IgY and IgY-Fc mutants were biotinylated to allow detection of MQ-NCSU cell binding using streptavidin conjugated to RPE (Dako cytomation, R0438). Proteins were dialysed against 0.1M sodium hydrogen carbonate, pH 9.0, using slide-a-lyser units (Pierce, 69570) and adjusted to ~1mg/ml. NHS-biotin (Sigma, H1759) was prepared as a 1mg/ml stock in DMSO (Sigma, D2650) and incubated with protein at a ratio of 3:1 (biotin to protein), for four hours at room temperature.

Concentration of proteins

IgY-Fc mutants were concentrated and buffer exchanged from affinity chromatography eluates using stirred cells and centrifugal filter units at 4°C, following the manufacturer's protocols. For large volumes (>100ml), an Amicon stirred cell with a 10,000 MWCO ultrafiltration membrane was used (Millipore, PLGC07610). For smaller volumes, Centricon Plus-70 10,000 MWCO (Millipore, UFC701008) or Amicon Ultra-15 5,000 MWCO (Millipore, UFC900524) filter units were used. Filters were thoroughly washed with TBS before use.

Crystallisation of IgY-Fc

Hanging drop vapour diffusion kits 'Classics' (Nextal, NSO-96-CLs) and 'PEGs' (Nextal, NSO-96-PGs), were used to screen for (papain-derived) IgY-Fc crystallisation conditions, following the manufacturer's protocol. Each well of the hanging drop plates contained a reservoir of crystallising buffer (typically, buffer, salt and precipitant). In each well, 1μl of IgY-Fc (~5mg/ml) was mixed with an equal volume of crystallisation buffer and sealed airtight with the drop suspended above the reservoir. This method causes the buffer in the droplet to slowly equilibrate with the reservoir. Under favourable conditions, this can lead to supersaturation and crystallisation of the protein. Plates were examined every day for one month, then once per week. Crystals were assayed for protein by adding 0.05% methylene blue, incubating for 5 minutes and examining for dye uptake using light microscopy. Optimisation of positive hits was performed by varying the crystallisation conditions (pH and/or concentration of salt and precipitant) in replicate screens (see chapter 3, table 3.5 ii). Common cryoprotectants were screened by examining crystal stability upon their addition.

Crystallisation conditions for recombinant IgY-Fc were screened using a sitting drop method. The mechanism for this method was similar to that described above (vapour diffusion), except that 100nl drops of IgY-Fc (~5 mg/ml) were dispensed onto sitting drop plates using a Cartesian Microsys robot (Genomic Solutions) and mixed with an equal volume of a large variety of screening solutions prepared by the Oxford Protein Production Facility. Plates were stored in a temperature controlled vault (TAP HomeBase) and monitored using an automated Veeco imager.

Deglycosylation under native conditions

Native serum IgY, IgY-Fc Cυ2-4 [wt] and human IgG1 were deglycosylated using PNGase F (New England Biolabs, P0704S). 20μg of protein was incubated with 1000 units (2μl) PNGase F in 20μl 1X G7 buffer (supplied with enzyme: 50mM sodium phosphate buffer, pH 7.5) for 72 hours at 37°C. Control reactions were also prepared as above, but without enzyme. Samples were assayed for complete removal of carbohydrate by SDS-PAGE.

Dynamic light scattering

Dynamic light scattering (DLS), also known as photon correlation spectroscopy, was used to compare thermal stability of several antibodies and antibody fragments. 0.1mg/ml protein solutions in DLS buffer were heated in 5°C increments from 20°C (allowing 3 minutes for equilibration at each temperature) using a DynaPro 99 instrument (Protein Solutions). An approximate melting temperature (T_m) was determined by thermally-induced aggregation. Although a DLS instrument does not measure melting or unfolding of proteins directly, the formation of aggregates can be inferred from rapid changes in the light scattering properties of the solution. DLS essentially measures how a beam of monochromatic light is differentially scattered over time. This can be used to calculate the rate at which particles in solution are moving (i.e. Brownian motion), assuming they can be modelled as spheres of equivalent and homogeneous shape and density. In this case, these particles are proteins. The mean hydrodynamic radius (Stoke's radius) of a protein solution increases as the proteins unfold and aggregate, which affects their motion in solution, as defined by the Stokes-Einstein equation:

$$D = \frac{k_B T}{3\pi\eta d} \qquad \text{(eqn 2.3 i)}$$

where D is the particle diffusion constant, *d* is their diameter, T is the temperature (in Kelvin), η is the viscosity of the medium and k_B is Boltzmann's constant.

Enzyme linked immunosorbent assay (ELISA)

An anti-IgY 'sandwich' ELISA was used to estimate the concentration of recombinant IgY-Fc in transfected cell culture supernatant. All wash steps were carried out using PBS-Tween with a SkanWasher 300B automated ELISA washer (Skatron Instruments). 96-well Maxisorp plates (Nunc, 442404) were coated with 100µl donkey anti-IgY (Chemicon, AP194) capture antibody (at 140ng/ml in ELISA coating buffer) overnight at 4°C. Plates were washed twice, then uncoated sites were blocked with 200µl PBS-Tween-Marvel for 1 hour at room temperature. Plates were washed twice again, then 100µl of each sample (serially diluted in fresh culture medium) and control (fresh medium alone) were added in triplicate. On every plate, three-fold serial dilutions of purified papain-derived IgY-Fc (starting at 800ng/ml in culture medium) were also added in triplicate, in order to generate a standard calibration curve. Plates were incubated overnight at 4°C, then washed 4 times. Rabbit anti-IgY-HRP (Promega, G135A) was added to every well (100µl per well, 0.5µg/ml in PBS-Tween-Marvel), and incubated for 4 hours at room temperature. Plates were washed 4 times and developed by adding 50µl 0.4mg/ml OPD (Sigma, P-6912-100TAB) in ELISA phosphate-citrate buffer for 10-20 minutes in the dark. HRP

catalyses a colourimetric reaction between OPD and hydrogen peroxide (generated by hydrolysis of sodium perborate), so can be used to measure the concentration of anti-IgY, and thus IgY-Fc, present in each well. The reaction was stopped by adding 50µl 3M HCl to each well. The absorbance at 492nm (A_{492}) of each well was determined using a Multiscan EX plate reader (Thermo) and the standard curve was plotted using Ascent software v2.6 (Thermo). The concentration of each sample was estimated from the linear part of the curve, using a sample dilution that lay within the appropriate sensitivity range.

Ellman assay

A modified Ellman assay (Ellman, 1958; Riddles *et al.*, 1979) was used to quantify free sulfhydryls in IgY and IgY-Fc, in order to establish whether all cysteine residues in the upsilon heavy chain are involved in disulfide bonds. Ellman's reagent, DTNB (Sigma, D8130), reacts with free thiol groups, so that an equimolar quantity of 2-nitro-5-thiobenzoate (TNB) is bound. Upon reduction, the coloured anion (TNB^{2-}) is released and can be detected photometrically, allowing the molar ratio of protein:free sulfhydryls to be determined. Bovine serum albumin (which contains one free sulfhydryl) was used as a positive control. Human IgG1 and IgG1-Fc (which contain no free sulfhydryls) were used as negative controls. DTNB was freshly prepared at 4mg/ml (in 100mM phosphate buffer, pH 7.2, + 0.1mM EDTA) and 6µl was added to 200µl of sample protein at ~1mg/ml in PBS + 6M guanidine-HCl. Each sample was incubated for 15 minutes at room temperature, then dialysed into PBS (with 0.1% sodium azide) using slide-a-lyser units (Pierce, 69570). The absorbance of each sample at 280nm and 412nm was measured using a Cary 50

spectrophotometer (Varian), before and after the addition of 0.05% β-mercaptoethanol. The concentration of protein was determined using A_{280} absorption coefficients calculated by ProtParam software (see below). The concentration of TNB^{2-} was determined using an A_{412} absorbance coefficient of 14,150 $M^{-1}cm^{-1}$ (Riddles *et al.*, 1979).

Estimation of protein concentration

The concentration of protein in a solution was determined using UV spectrophotometry. The absorbance of a sample in a 1cm path length quartz cuvette (Hellmann) was measured across a spectrum of UV wavelengths (240 to 340 nm) using a Cary 50 spectrophotometer (Varian). The absorbance of the buffer used in the sample was also measured across the same spectrum and subtracted from the sample absorbance. The Beer-Lambert law defines the correlation between the absorbance, A, and concentration, c:

$$A = c\varepsilon l \qquad \text{(eqn 2.3 ii)}$$

where l is the path length (cm) and ε is the absorbance coefficient ($M^{-1}cm^{-1}$). The absorbance coefficients for IgY and the IgY-Fc mutants (table 2.3 iii) were calculated using ProtParam software (Walker, 2005).

		Molar Extinction Coefficient	Molecular Weight (kDa)	A_{280} (1mg/ml)
	Whole Y	209125	161.94	1.29
IgY-Fc Cu2-4	24wt	90965	73.66	1.24
	N308Q	90965	73.77	1.23
	N308Q N407Q	90965	73.8	1.23
IgY-Fc Cu3-4	34wt	62630	50.95	1.23
	C340S	62505	50.91	1.23
	C347S	62505	50.91	1.23
	C340S C347S	62380	50.88	1.23

Table 2.3 iii
Calculated molecular weight and UV absorbance characteristics of IgY and IgY-Fc

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Ion exchange chromatography

A DEAE sepharose ion exchange column (GE Healthcare, 17-5154-01) and an FPLC chromatography system (Pharmacia) was used to separate IgY-Fc fragments from other yolk IgY papain digestion products. Proteins were bound to the column and differentially eluted on the basis of their charge in different ionic strengths of buffer. The flow rate was set to 0.5ml/min and the column equilibrated using 50ml IE bind / wash buffer. ~10mg of IgY papain digest (dialysed into IE bind / wash buffer) was loaded onto the column, washed with 25ml IE bind / wash buffer, and eluted using a salt gradient (i.e. a steady increase from 0 to 100% IE elution buffer). Fractions were collected across A_{280} peaks.

N-terminal protein sequencing

N-terminal amino acid sequencing was performed by J.N. Keen, University of Leeds, using Edman degradation and reverse-phase HPLC.

Papain digestion

Enzymatic cleavage of IgY purified from chicken egg yolk was used to prepare IgY-Fc fragments. Papain (Sigma P3125) was pre-activated by incubation in papain digestion buffer (pH 5.0) for 15 minutes at 37°C on a rotator. Papain was added to IgY (~1mg/ml) in papain digestion buffer (pH 7.0), using an enzyme:substrate ratio of 1:25 (w/w), and incubated for 16 hours at 37°C on a rotator. The reaction was stopped by adding iodoacetamide (Sigma I-1149) to a final concentration of 30mM. IgY-Fc was isolated using a combination of ion exchange and size exclusion chromatography.

Purification of IgY from chicken egg yolk

Chicken egg yolk IgY was isolated using an Eggstract Kit (Promega, G1531), following the manufacturer's protocol. Repeated lipid and protein precipitation steps were used to separate IgY, which was finally resuspended at ~2-3mg/ml in PBS with 0.05% (w/v) sodium azide. IgY was sterile filtered using a 0.2µm membrane (Sartorius, 16534----------K) and stored at 4°C.

Radioiodination of antibodies and antibody fragments

Affinity purified IgY and IgY-Fc were radiolabelled with ^{125}I for use in MQ-NCSU cell binding assays using the chloramine-T method (McConahey and Dixon, 1980). 1mg/ml chloramine-T (Sigma, 857319) was freshly prepared in

100mM sodium phosphate buffer, pH7.0. 5µl chloramine-T solution was added to a mixture of 7.4MBq ^{125}I and 200µg IgY or IgY-Fc (~1mg/ml in PBS) every 150 seconds for 10 minutes. The reaction was stopped by the addition of 20µl 1mg/ml sodium metabisulfate (Sigma, 255556) in 100mM sodium phosphate buffer, pH7.0. Desalting spin columns (Pierce, 89849) were used to remove unincorporated ^{125}I and exchange the radiolabelled IgY or IgY-Fc into PBS, following the manufacturer's protocol. The specific activity of each radioligand was determined by estimation of the protein concentration (by UV spectrophotometry) and radioactivity (counts/min), using a Wizard 1480 gamma counter (Perkin-Elmer).

Reduction and alkylation of antibodies and antibody fragments

The susceptibility of inter-heavy chain disulfide bonds to reduction was investigated using dithiothreitol (DTT). Before analysis using SDS-PAGE, samples (at 0.5mg/ml in PBS) were incubated with a two-fold serial dilution of fresh DTT (9 concentrations from 10mM to 0.04mM) for 1 hour at room temperature. 200mM iodoacetamide (Sigma, I-1149) was added to alkylate reduced cysteines and to prevent further reduction upon protein denaturation in SDS-PAGE loading buffer.

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), based on the method of Laemmli (Laemmli, 1970), was used to assess the purity of protein solutions, to estimate the molecular weight of proteins, and to prepare samples for N-terminal sequencing. SDS is an anionic detergent,

which causes proteins to adopt a net negative charge, allowing them to migrate toward the anode in an electric field. By forcing them to move through a polyacrylamide gel, proteins can be separated on the basis of their size or relative molecular weight.

Gels were prepared using an appropriate percentage acrylamide, in order to ensure adequate size separation of sample proteins (table 2.3 iv).

Percentage Acrylamide	Vol. acrylamide stock (ml)	Vol. 2M Tris pH 8.8 (ml)	Vol. 1M Tris pH 6.8 (ml)	Vol. 10% SDS (ml)	Vol. 10% APS (ul)	Vol. TEMED (ul)
10	8.33	4.7	-	0.25	160	40
12	10	4.7	-	0.25	160	40
15	12.5	4.7	-	0.25	160	40
Stacking gel	1	-	1.25	0.1	64	15

Table 2.3 iv
Composition of polyacrylamide gels

Gels were prepared using stock solutions shown to a final volume of 25ml using Milli-Q water (except the stacking gel, which was made to 10ml). TEMED was added last, immediately before pouring.

Gels were poured and set using Atta PAGE apparatus and placed in an Atta gel tank with 1X SDS-PAGE running buffer in the upper and lower reservoirs. Protein samples were diluted into SDS-PAGE loading buffer (20-30µl final volume), with or without 2% β-mercaptoethanol (for a reduced or non-reduced gel, respectively). Samples were heated at 100°C for 5 minutes, centrifuged for 1 minute in a microfuge and loaded onto the gel adjacent to

appropriate molecular weight markers. The gel was run at 150V for ~1-2 hours, until loading dye had reached the bottom of the gel plate. Gels were removed and stained with either Coomassie blue (total protein) for 1 hour, followed by destain overnight, or with a Periodic acid-Schiff (PAS) stain, using a Gelcode kit (Pierce, 24562), following the manufacturer's instructions.

Size exclusion / gel filtration chromatography

Size exclusion or gel filtration chromatography was used to estimate the molecular weight of IgY and IgY-Fc mutants under non-denaturing conditions and to purify material for crystallisation. A Superdex 200 column (GE Heathcare, 17-5175-01) connected to a Gilson HPLC system was equilibrated and run using HPLC running buffer at a flow rate of 0.75ml/min. The column was calibrated using a series of protein standards of known molecular weight (Sigma, MW-GF-1000). Protein samples, either 60μl at ~0.1mg/ml (for analytical runs) or 500μl at ~1mg/ml (for preparative runs), were injected onto the column, and the UV absorbance (A_{280}) of the eluate monitored over 40 minutes.

Large proteins and aggregated material elute in a smaller volume (i.e. sooner) than smaller proteins, which are retarded by their entry into pores in the gel matrix.

Semi-dry transfer of proteins

Proteins were transferred from SDS-PAGE gels onto either (a) PVDF membrane (pre-activated by washing in methanol and equilibrated in semi-dry transfer anode buffer), for N-terminal sequencing or (b) nitrocellulose membrane, for Western blotting, using a Novablot II semi-dry electroblotter

(Pharmacia). In both instances, the membrane and four pieces of filter paper (Whatman No. 1) were cut to the size of the gel. A stack was assembled comprising two pieces of filter paper (soaked in semi-dry transfer anode buffer), membrane, gel, and two more pieces of filter paper (soaked in semi-dry transfer cathode buffer). Protein transfer occurs in the direction cathode to anode, so the stack was positioned membrane toward the anode, gel toward the cathode. Any air bubbles between the gel and membrane were removed and the blotter run at 0.8-1 mA/cm^2 for 30-45 minutes.

Western Blot

Western blotting was used to detect IgY and IgY-Fc fragments transferred onto a nitrocellulose membrane from an SDS-PAGE gel. An appropriate quantity of sample was loaded so that the quantity of detected protein(s) would fall within the sensitivity of the technique (5-100ng, depending on sample purity and efficiency of transfer). Following adsorption onto the blotting membrane, unbound sites were blocked by incubating for 60 minutes in PBS-Marvel at room temperature, with gentle shaking. The blocking solution was then discarded and the membrane was incubated in 0.5µg/ml rabbit anti-IgY (Promega, G135A) in PBS-Tween-Marvel for a further 60 minutes. The membrane was washed three times by incubating in PBS-Tween for 10 minutes each, then rinsed with PBS. The presence of anti-IgY-HRP was revealed using an HRP-catalysed chemoluminescence reaction (Luminol / H_2O_2, Pierce, 434080). The membrane was used to expose a piece of Fuji RX X-ray film (GRI, JTS010) for 20 seconds. The film was developed using an X-OMAT

developer (Kodak). The membrane was used to expose another piece of film for a further ~16 hours to ensure no further bands were visible.

2.4 Cell methods

Buffy coat layer separation

Chicken peripheral blood was collected into acid citrate dextrose (ACD) anticoagulant solution (Sigma, C3821), using ~0.25ml ACD per ml of blood. The Buffy coat (leukocytes) was separated by centrifugation at 200xg (without the brake) and removed using a Pasteur pipette. Cells were resuspended in PBS and kept on ice.

Cell binding assays

Radioiodinated IgY and IgY-Fc fragments were used to determine the number of IgY-Fc receptors on MQ-NCSU chicken monocytes and their binding kinetics. Binding was assumed to follow a simple one-step reaction:

$$[R] + [L] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [RL] \qquad \text{(eqn 2.4 i)}$$

where [R] is the concentration of Fc receptor, [L] is the concentration of ligand (IgY or IgY-Fc), [RL] is the concentration of receptor:ligand complex, k_{+1} is the association rate constant (or on-rate) and k_{-1} is the dissociation rate constant (or off-rate). In cell binding assays, measurement of bound ligand allows [RL] to be determined.

The mean number of total receptor sites per MQ-NCSU cell was determined by saturating with radiolabelled IgY or IgY-Fc Cυ2-4 [wt] of known

specific activity (see section 2.3, 'Radioiodination of antibodies and antibody fragments'). MQ-NCSU cells growing in IgY-free medium were washed and incubated in PBS-BSA (with 0.05% sodium azide) for 10 minutes at 4°C. $1x10^6$ cells were incubated with 10, 20 and 75nM ^{125}I labelled IgY or IgY-Fc Cυ2-4 [wt] in 100μl PBS-BSA (with 0.05% sodium azide) for 2 hours at 4°C, then layered onto a 200μl cushion of phthalate oil in a microtube and spun for 1 minute in a microfuge to separate cells (and therefore bound radioligand) from supernatant containing free ligand. The bottom of the tube was cut off so that the radioactivity of the cell pellet could be measured using a Wizard 1480 gamma counter (Perkin-Elmer). No more radioligand was bound when cells were incubated with 75nM than with 20nM, so receptors were assumed to be saturated.

Dissociation experiments were performed using the method of Kulczycki and Metzger (Kulczycki and Metzger, 1974), but with all steps carried out at room temperature. MQ-NCSU cells growing in IgY-free medium were washed and incubated in PBS-BSA (with 0.05% sodium azide) for 10 minutes and incubated at $1x10^7$ cells/ml with 10nM ^{125}I-lablled IgY or IgY-Fc for 1 hour on a rotator. The cells were spun for 2 minutes in a microfuge, resuspended at the same cell concentration in PBS-BSA (with 0.05% sodium azide) containing excess (350nM) unlabelled IgY and placed back on the rotator. At 1, 2, 3, 4, 5, 10, 20, 40 and 60 minute time points, 100μl ($1x10^6$ cells) were removed, immediately spun through phthalate oil and the cell pellet kept for gamma counting, as described above. Non-specific binding was determined by pre-incubating cells in excess (350nM) IgY for 60 minutes before addition of 10nM

radiolabelled ligand for a further 60 minutes. Non-specific counts were subtracted from the counts obtained from the unblocked cells.

The rate of change of a receptor:ligand complex is determined by the rate of its formation minus the rate at which dissociation occurs:

$$\frac{d[RL]}{dt} = k_{+1}[R][L] - k_{-1}[RL] \qquad \text{(eqn 2.4 ii)}$$

As radioligand dissociation was measured in the absence of re-association (due to the presence of excess unlabelled ligand), the $k_{+1}[R][L]$ component in equation 2.4 ii is zero. In these experiments, the change in receptor:ligand concentration is entirely determined by the rate of dissociation:

$$\frac{d[RL]}{dt} = -k_{-1}[RL] \qquad \text{(eqn 2.4 iii)}$$

A non-linear regression analysis was made by fitting an equation that expands on equation 2.4 iii (Goodrich and Kugel, 2007), in order to allow for populations of radioligand that dissociate very quickly or very slowly (e.g. caused by non-specific interactions or aggregated radioligand):

$$[RL]_t = ([RL]_{max} - [RL]_{min})(e^{-k_{-1}t}) + [RL]_{min} \qquad \text{(eqn 2.4 iv)}$$

where $[RL]_{max}$ is the true starting point of the decay curve and $[RL]_{min}$ is where it levels out (i.e. no further radioligand dissociates). SigmaPlot v6.0 (SPSS) was used to fit this equation to the data, using $[RL]_{max}$, $[RL]_{min}$ and k_{-1} as variables. The results obtained using equation 2.4 iv were checked against dissociation

rate constants determined from linear regression of a plot of ln[RL] versus time (Rosenthal, 1967); equation 2.4 iii can be derived to show that the gradient of this plot is equal to k_{-1}. However, this type of analysis can frequently produce errors, so was only used for rough comparison with values obtained using non-linear regression, which is a more appropriate means of analysis in this type of study (Bylund and Toews, 1993).

Association experiments were performed following the method of Young *et al.* (Young *et al.*, 1995). A similar protocol to that used for dissociation experiments was used, except that cells were removed at 1, 2, 3, 4, 5, 10, 20, 40 and 60 minutes after the addition of 10nM radiolabelled IgY or IgY-Fc. Non-specific binding was determined by pre-blocking with excess unlabelled ligand, and used to determine specific binding, as described above. The data were analysed by fitting an equation derived by Young *et al.* (1995):

$$[RL] = \left[\frac{k_{+1}[L][R]}{k_{+1}[L] + k_{-1}}\right] - \left[\frac{k_{+1}[L][R]}{k_{+1}[L] + k_{-1}}\right] e^{-(k_{+1}[L] + k_{-1})t} \qquad \text{(eqn 2.4 v)}$$

Equation 2.4 v assumes that ligand is present in excess, so that the concentration, [L], is effectively constant. The equation was fitted to the data using SigmaPlot v6.0 (SPSS), using k_{+1} and k_{-1} as variables. The total receptor concentration [R] was calculated from the estimated total number of receptors per cell and the number of cells per unit volume. The concentration of receptor:ligand complex, [RL], was calculated from the bound counts per cell per unit volume, divided by the specific activity of the radioligand.

Cell culture

See section 2.1.2 for cell lines and media used. All cell lines were maintained using Nunc tissue culture plastics or (for use in fluorescence microscopy) grown on glass chamber slides (Fisher, TKT-210-150R), at 37°C in an air-jacketed incubator (Nuaire) supplemented with 5% carbon dioxide. Cells were split 1:5 upon confluency. CHO-K1 and RBL-SX38 cells were detached by exchanging the culture media for trypsin (Sigma, T4174) in PBS for 5-10 minutes, then washing cells in fresh media. Cell stocks were stored at $1x10^7$ cells per ml of freeze medium in cryovials, which were incubated for 24 hours at -80°C, then transferred to liquid nitrogen.

Density gradient separation of chicken blood basophils

Percoll (Amersham, 17-0891-01) was prepared at a density of 1.080g/ml in order to separate chicken peripheral blood basophils by mixing 20ml stock isotonic Percoll with 13.1ml 1.5M sodium chloride. Buffy coat layer cells in PBS were layered onto the Percoll gradient and centrifuged at 400xg for 20 minutes (without the brake). Basophils were collected below the Percoll layer, other leukocytes remained above.

Estimation of cell number and viability

Cell counts were estimated using a haemocytometer. An aliquot of cells were diluted in PBS and Trypan blue solution (to final concentration of 0.2%), then 10µl were loaded into a haemocytometer. When the coverslip is placed on the haemocytometer with Newton's rings visible, the primary square contains $1x10^{-4}$ cm^3 of cell suspension. The cell concentration of the original sample can then be calculated as follows:

Cells per ml = (N° of cells in primary square) x 10^4 x (dilution factor)

Live cells exclude Trypan blue dye, whereas dead cells take it up readily, which allows viability to be estimated.

Flow cytometry (FACS)

Flow cytometry or fluorescence activated cell sorting (FACS) was used to detect immunostained cells following their incubation with specific antibodies and/or antibody conjugates. In all experiments, cells were washed (by centrifugation at 1000xg for 5 minutes) and incubated for 10 minutes in PBS-BSA before immunostaining. Cells were washed twice with PBS-BSA both before and after all antibody incubation steps.

A FACSAria preparative instrument (BD Biosciences) was used to separate a highly fluorescing population of chicken buffy coat layer cells, following incubation for 1 hour at 4°C with anti-IgY-FITC (10μg/ml in PBS-BSA).

For all other experiments a FACSCalibur analytical instrument (BD Biosciences) was used. For extracellular staining, PBS-BSA was used as a blocking, washing and incubation buffer. Cells were fixed and analysed in PBS + 4% formaldehyde (Sigma, F1635) only after all immunostaining steps, which were carried out for 1 hour each at 4°C. For intracellular staining, cells were fixed first, then washed and pre-incubated with PBS-BSA + 0.1% saponin (Sigma S4521) for 30 minutes at room temperature. All subsequent immunostaining steps were also carried out in PBS-BSA + 0.1% saponin for 30 minutes at room temperature.

In all experiments, the concentration of antibody used was titrated in order to optimise the signal-to-noise ratio. In general, ~20μg/ml (polyclonal) or ~5μg/ml (monoclonal) was used for primary antibodies, and ~10μg/ml was used for secondary antibodies conjugated to fluorophores.

Fluorescence microscopy

The cell surface expression of N-terminal V5-tagged chFcR/L was detected using transiently transfected CHO-K1 cells grown on chamber slides. Growth medium was removed and cells were incubated in PBS-BSA for 15 minutes, followed by FACS fix for 10 minutes. Before and after all immunostaining steps, cells were washed by incubating in PBS-BSA for 15 minutes, three times. Cells were immunostained using 10μg/ml mouse monoclonal anti-V5 antibodies (Sigma, V8012) followed by 50μg/ml anti-mouse-FITC antibodies (Dako, F0479) for 30 minutes each at room temperature in PBS-BSA. Slides were mounted with fluorescence mounting medium (Dako, S3023) and analysed using an Axioskop 20 (Zeiss) fitted with filter set 09 (Zeiss, 488009-0000-000) and an Axiocam CCD.

Giemsa staining

Chicken leukocyte types were identified using Giemsa stain . Cells were smeared or spun onto glass slides using a cytospin centrifuge (Shandon), air-dried and fixed with methanol. Slides were stained with Giemsa solution (Raymond A Lamb) for 20 minutes, then dipped in 1% acetic acid followed by distilled water. Slides were air-dried, mounted and examined by light microscopy.

Solubilisation of cell membrane proteins

Using a similar method to those reported for solubilisation of the high affinity receptor for IgE (Conrad *et al.*, 1976; Kinet *et al.*, 1985), MQ-NCSU cell membrane proteins were solubilised in non-ionic detergent (Nonidet P-40 or Triton X-100). Cells were resuspended at 2 x 10^7 cells/ml in cold lysis buffer and homogenised using a 21G needle. Cells were sonicated using five bursts of 1 second each, incubated on ice for 30 minutes (vortexing every 5 minutes), then centrifuged at 20,000xg for 20 minutes at 4°C.

Specific production rate

The IgY-Fc production rate of transiently transfected CHO-K1 cells or stably transfected NS-0 cells was determined using an anti-IgY ELISA. Cells were plated at 5x10^5 cells/ml in fresh non-selective medium in a 24-well tissue culture plate and incubated for 24 hours. Supernatants were harvested by centrifugation and the concentration of IgY-Fc measured by ELISA (see section 2.3). Specific production rate was expressed in μg protein/1x10^6 cells/24 hours.

Stable transfection of HEK 293 cells

HEK 293 cells at ~70% confluency growing on 6-well plates were transfected with purified plasmid DNA using Trans-IT-293 reagent (Mirus, 2700), following the manufacturer's protocol. After 48 hours, cells were re-plated onto 100mm tissue culture dishes in growth medium supplemented with an appropriate antibiotic, depending on the transfected vector. The concentration of antibiotic used (either 0.9mg/ml G418 or 0.3mg/ml hygromycin B) was the minimal concentration necessary to kill 100% of untransfected cells

within 10 days, as determined by a killing curve experiment. Cells were incubated for 10-20 days until colonies of stable transfectants were visible. Individual colonies were isolated using cloning cylinders (Sigma, C7983-50E), propagated in selective media and assayed using immunofluorescent staining for intracellular and extracellular expression.

Stable transfection of NS-0 cells

IgY-Fc vectors were linearised by restriction digestion with SalI, purified by phenol:chloroform extraction and used to transfect NS-0 cells by electroporation. Briefly, 40μg DNA (resuspended in 50μl sterile Milli-Q water) was mixed with $1x10^7$ cells (washed once with ice cold PBS, then kept on ice) in an ice cold electroporation cuvette, then pulsed twice (at 1500V, 3μF) and immediately returned to ice for 5 minutes. Cells were added to 30mls warm non-selective NS-0 medium and 10mls removed to create three serial dilutions. All dilutions were plated onto 96-well plates (50μl per well) and incubated for 24 hours. In order to select stable transfectants expressing glutamine synthetase (encoded by the transfected vector), 150μl selective media was added per well and cells were incubated for 20-30 days, until colonies were visible. Individual colonies were propagated and repeatedly screened for IgY-Fc expression using an anti-IgY ELISA.

Transient transfection of CHO-K1 cells

Transient transfection of CHO-K1 cells was performed on cells at ~70% confluency, growing in either 24-well plates or on chamber slides. Purified plasmid DNA was used with Trans-IT-CHO reagent (Mirus, 2170), following the

manufacturer's protocol. Cells transfected with chFcRγ and/or chFcR/L vectors were analysed using immunostaining and fluorescence microscopy after 48 hours. Cells transfected with IgY-Fc vectors were counted and resuspended to $5x10^5$ cells/ml in fresh growth medium after 48 hours in order to perform a specific production rate assay.

Chapter 3

Purification of Yolk IgY, enzymatic digestion and growth of IgY-Fc crystals

3.1 Overview

Comparisons between the structure and function of chicken IgY and mammalian IgG and IgE, which are thought to have originated from an IgY-like ancestor (Warr *et al.*, 1995), may allow us to gain insights into the evolution of these important isotypes. Much of what we know about the structure of IgY to date is predicted from the upsilon (υ) heavy chain cDNA sequence (Parvari *et al.*, 1988), as a crystal structure has not yet been solved. The apparent similarities between IgY and IgE are of particular interest due to the unique structure of IgE, and the role this plays in exacerbating the hypersensitive response (Gould *et al.*, 2003).

The structures of the incomplete (Cε3-4) and complete (Cε2-4) Fc region of IgE have been solved (Wan *et al.*, 2002; Wurzburg *et al.*, 2000), as have several IgG structures (Harris *et al.*, 1998; Huber *et al.*, 1976), so it will be possible to make detailed comparisons between these and an IgY-Fc structure.

Traditionally, mammalian IgG can be digested with papain (Porter, 1959), a cysteine protease, to separate the parts of the molecule that bind antigen (Fabs) from the part that bind receptors in order to initiate effector function (Fc). As the Ig domains that make up the Fc region are invariant for any given isotype, these fragments are crystallisable, and have been used in many X-ray diffraction studies. Papain digestion of IgE, however, appears to completely

degrade the Fc region (Haba and Nisonoff, 1991), so recombinant material for the crystal structure analysis had to be prepared by transfection of either insect or mammalian cells with expression vectors encoding domains Cε3-4 or Cε2-4 respectively (Garman *et al.*, 2000; Wan *et al.*, 2002; Wurzburg *et al.*, 2000; Young *et al.*, 1995).

Chicken IgY can also be cleaved into Fab and Fc using papain (Dreesman and Benedict, 1965a; Kubo and Benedict, 1969), although at the time of these experiments, the composition of the Fc was unknown.

Chicken egg yolk IgY was purified and digested with papain in order to explore whether IgY is cleaved in an IgG-like or IgE-like manner, with the aim of purifying IgY Fc for preparation of crystals.

3.2 Purification of yolk IgY

IgY is transferred into the chicken egg yolk in order to confer protection on the developing embryo, reaching a quantity of 60-100mg in the laid egg. For preparation of a yolk IgY stock, a single large egg provided sufficient quantity of antibody to accommodate losses upon purification and concentration. Initial precipitation of IgY from yolk yielded a heterogeneous mixture with some impurities, which were removed by HPLC size exclusion chromatography (Figure 3.2).

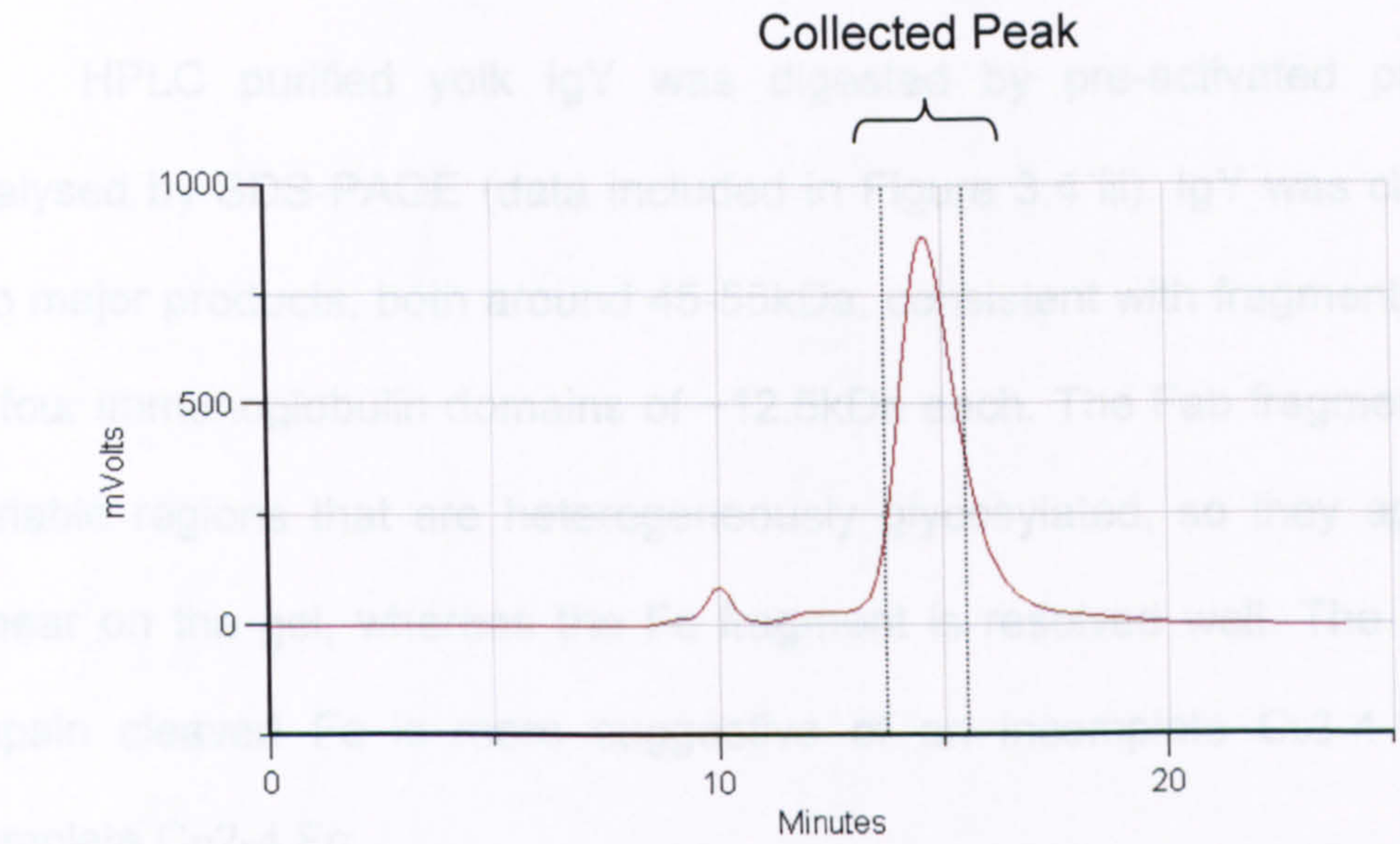


Figure 3.2

Purification of chicken egg yolk IgY by size exclusion chromatography

(A) Gel filtration profile indicating pooled fractions of chicken egg yolk IgY from Superdex 200 column.
(B) Non-reducing 10% SDS-PAGE (1 & 2) and anti-IgY western blot (3 & 4) showing IgY recovered from crude yolk precipitate (1 & 3) and post-HPLC purified material (2 & 4).

3.3 Papain cleavage of yolk IgY

HPLC purified yolk IgY was digested by pre-activated papain and analysed by SDS-PAGE (data included in Figure 3.4 iii). IgY was cleaved into two major products, both around 45-55kDa, consistent with fragments made up of four immunoglobulin domains of ~12.5kDa each. The Fab fragments contain variable regions that are heterogeneously glycosylated, so they appear as a smear on the gel, whereas the Fc fragment is resolved well. The size of the papain cleaved Fc is more suggestive of an incomplete Cυ3-4 Fc than a complete Cυ2-4 Fc.

3.4 Purification and characterisation of IgY-Fc

The products of papain digestion of yolk IgY were separated using ion exchange chromatography (Figure 3.4 i), followed by size exclusion chromatography (Figure 3.4 ii). 100pmol of the purified IgY-Fc (~5µg) were subjected to non-reducing SDS-PAGE and transferred to PVDF for N-terminal sequencing, which was performed and analysed by Dr. J.N. Keen (University of Leeds). The major sequence was found to begin SCSPIQL, with a small proportion of others differing by a few additional N-terminal residues. This sequence matches residues 346 to 352, which are found close to the N-terminus of Cυ3 predicted by Parvari *et al.* (1988), suggesting that the Fc fragment lacks the Cυ2 domains. The apparent molecular weight (Figure 3.4 ii) on non-reducing and reducing SDS-PAGE gels (~55kDa and ~30kDa respectively) is consistent with a disulphide linked Cυ3-4 Fc fragment (Figure 3.4 iv).

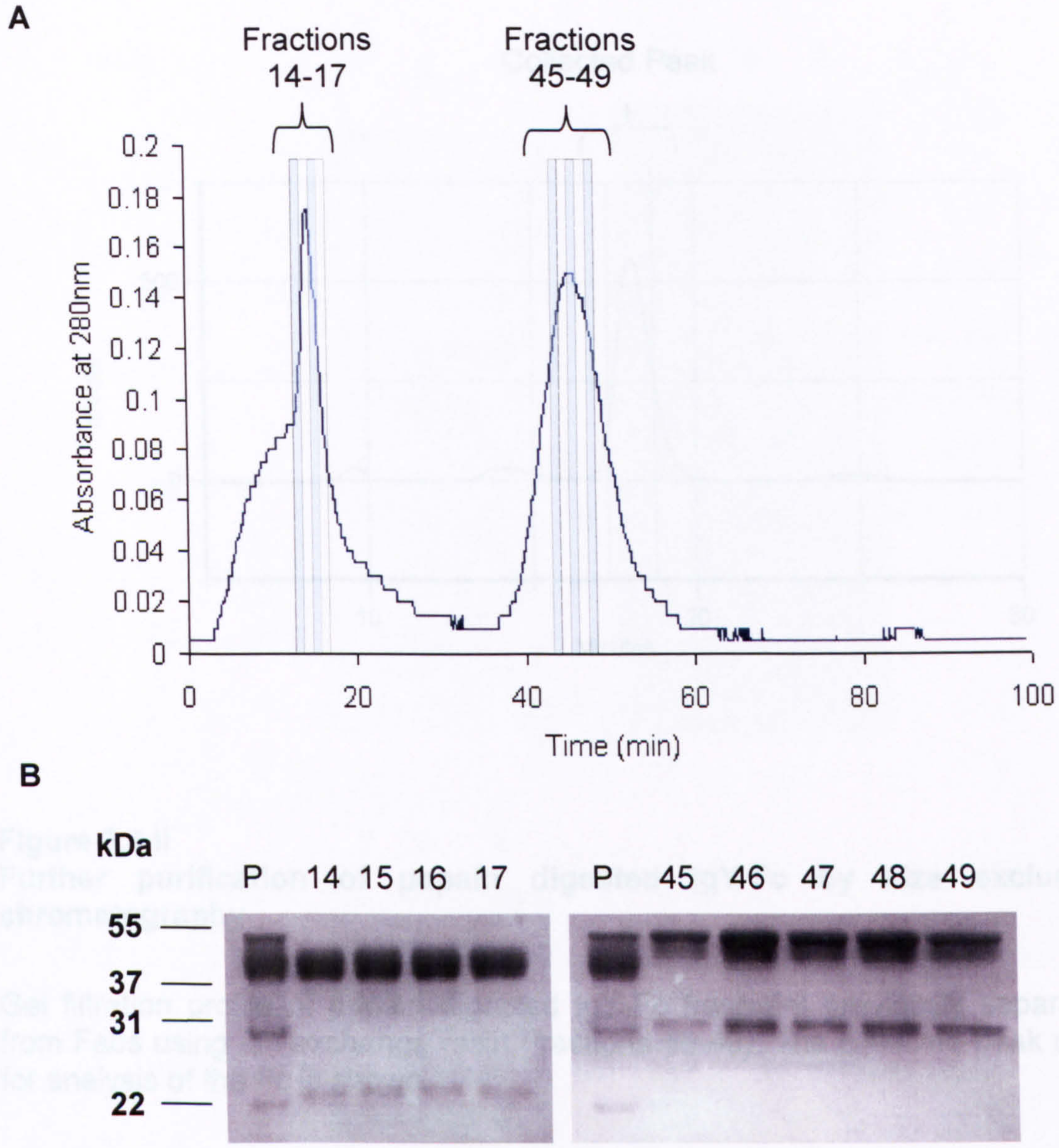


Figure 3.4 i
Separation of papain digested IgY fragments by ion exchange chromatography

(A) Elution profile of papain digested IgY fragments from a DEAE FF column showing collected fractions subsequently analysed (B) by non-reducing SDS-PAGE. P = papain digestion products before separation.

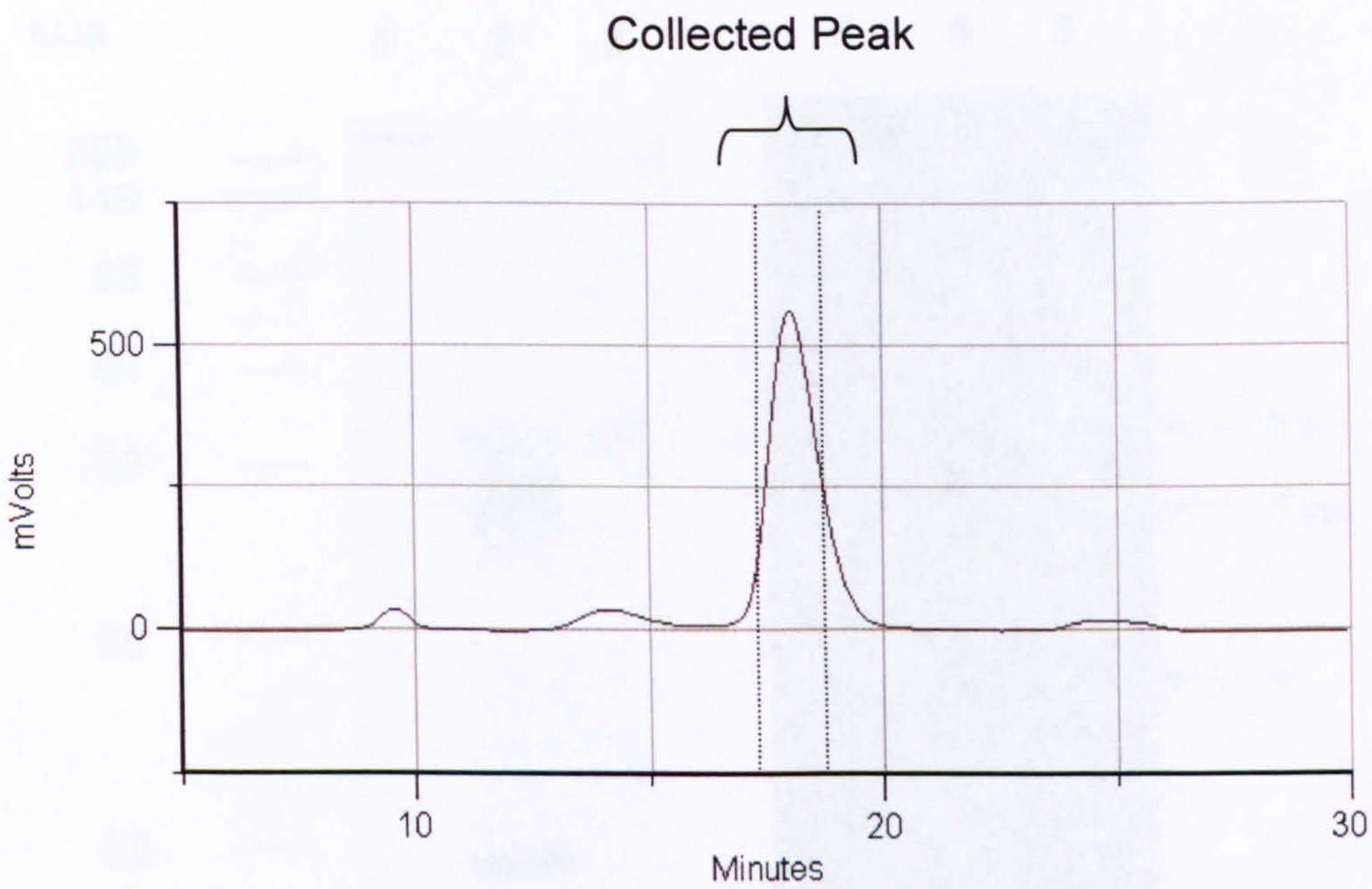


Figure 3.4 ii
Further purification of papain digested IgY-Fc by size exclusion chromatography

Gel filtration profile of papain digested IgY-Fc fragment previously separated from Fabs using ion exchange resin (fractions 45-49). The collected peak used for analysis of the Fc is shown.

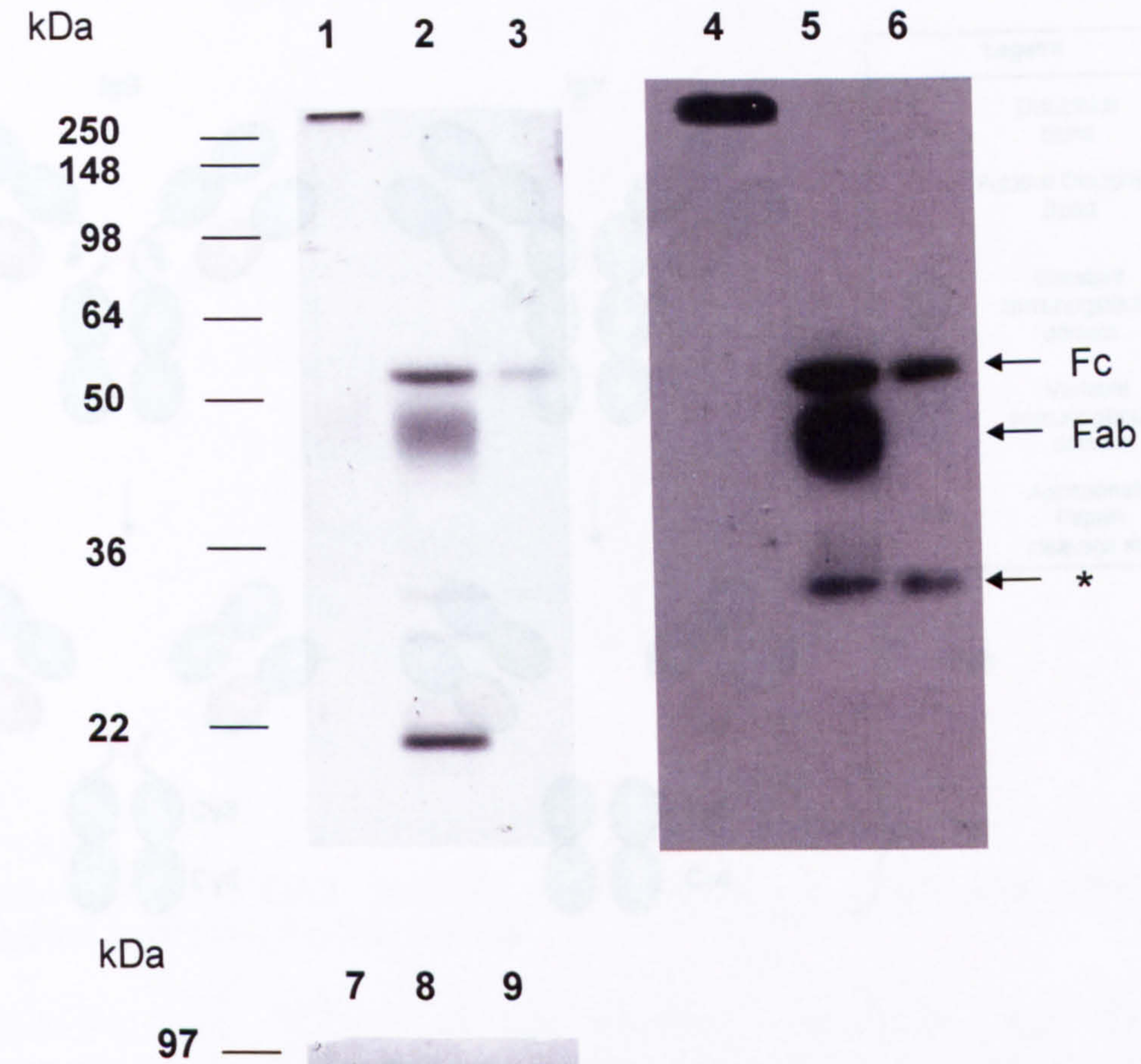


Figure 3.4 iii
Analysis of IgY papain digestion and purified Fc

Reducing (7,8 & 9, bottom) and non-reducing (1,2 & 3, top left) 12% SDS-PAGE gels and western blot (4,5 & 6, top right) showing stages of IgY-Fc preparation. Purified IgY (1, 4 & 7) was digested with papain (2, 5 & 8) and Fc separated using two chromatography techniques (3, 6 & 9). Bands consistent with Fab and Fc are marked. The other marked band (*) is a minor component of the separated Fc preparation and probably represents monomeric Fc as the preparation reduces into a single band (9). This phenomenon has been observed in preparations of IgE-Fc (Cε2-4) and is likely an artefact of SDS-PAGE.

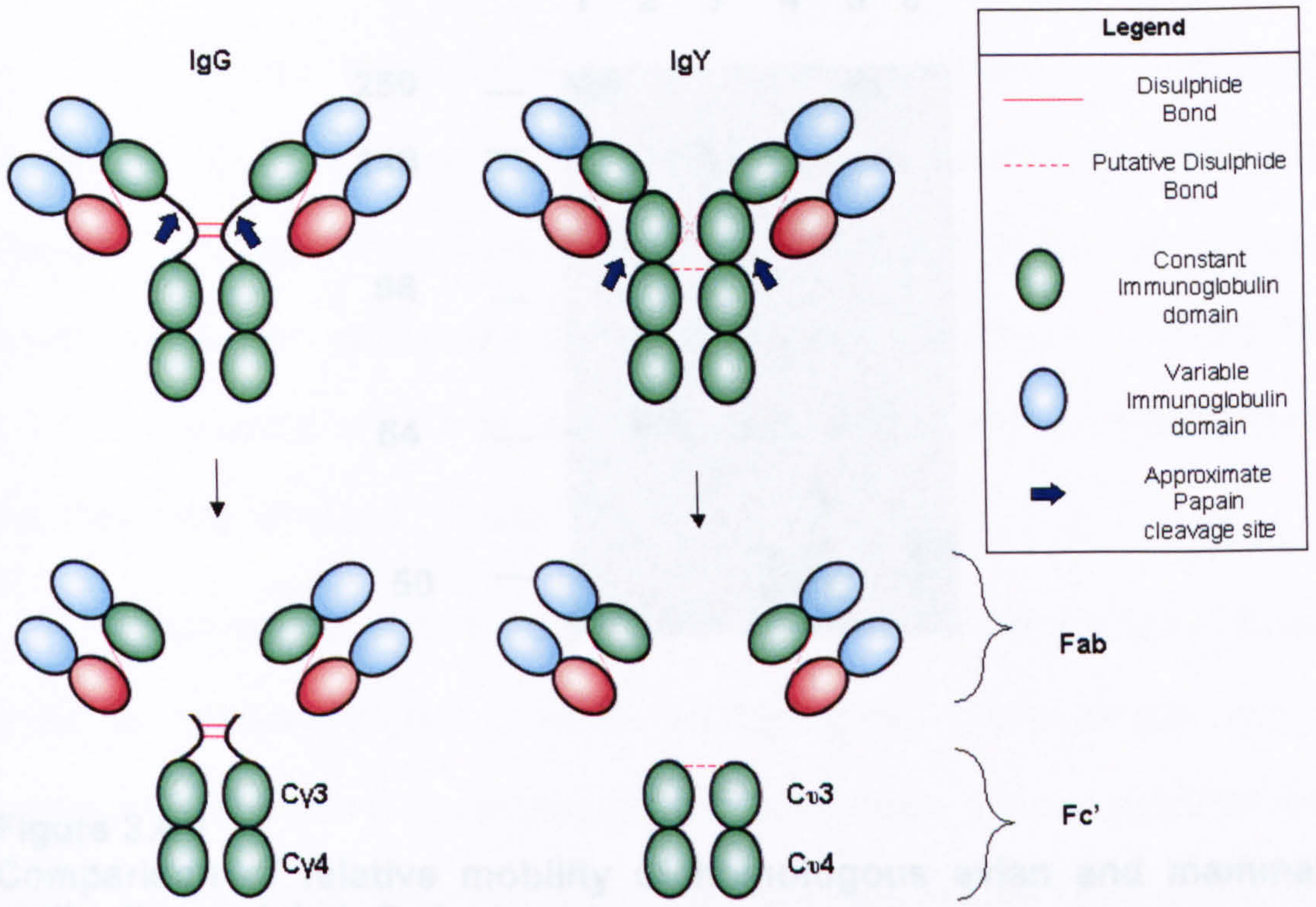


Figure 3.4 iv
Schematic of fragments produced by papain digestion of mammalian IgG and avian IgY

IgG is cleaved by papain in a specific manner to produce Fab and Fc fragments. The Fc fragments retain the interchain disulphide bonds that allow the Cγ2 and Cγ3 domains to remain dimeric. Papain cleavage of IgY also produces an Fc containing the orthologous Cυ3 and Cυ4 domains, despite the presence of an additional Cυ2 domain in the complete antibody, which appears to be completely degraded. The IgY-Fc remains dimeric after separation from the rest of the antibody, as does the IgG-Fc. Cysteine residues at the N-terminus of the IgY-Fc (position 347 in each chain) are apparently able to form an interchain disulphide bond.

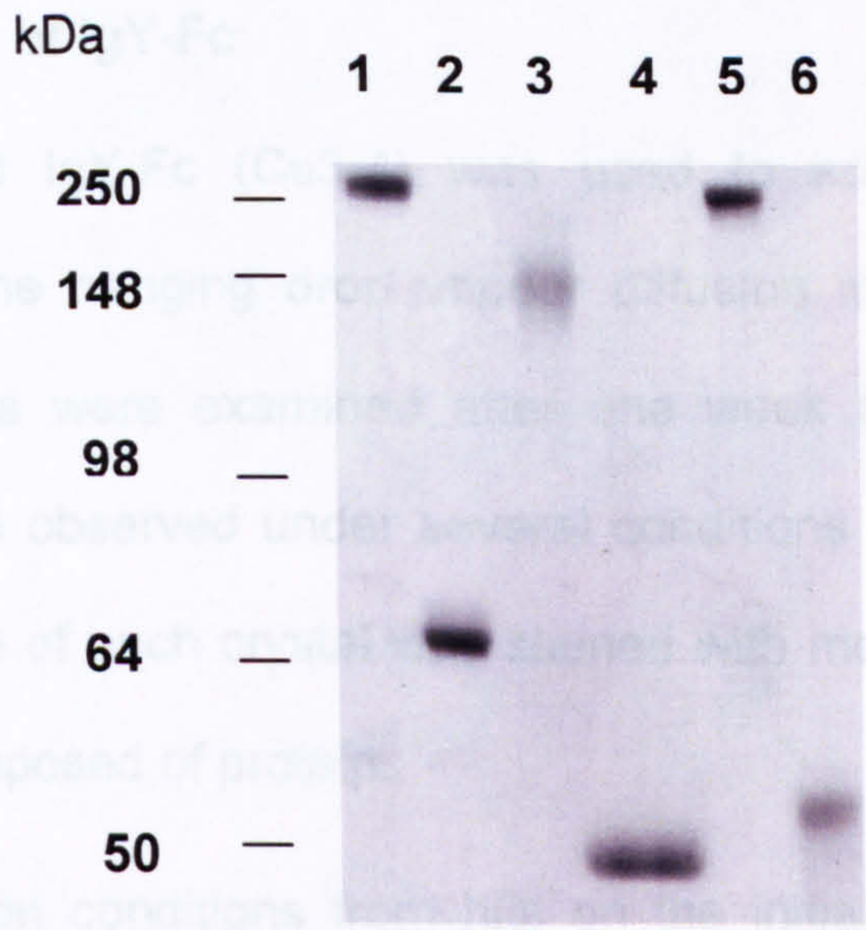


Figure 3.4 v
Comparison of relative mobility of homologous avian and mammalian antibodies and their Fc fragments

Non-reducing 9% SDS-PAGE gel showing mammalian IgE (lane 1), recombinant IgE-Fc Cε2-4 (lane 2), mammalian IgG (lane 3), papain produced IgG-Fc Cγ2-3 (lane 4), avian IgY (lane 5) and papain produced IgY-Fc Cυ3-4 (lane 6).

In the IgE-Fc, cysteine residues C241 and C328 in Cε2 are involved in interchain disulphide bonds (Wan *et al.*, 2002). The orthologous residues, C252 and C340 occur in Cυ2 and are therefore missing from the incomplete IgY-Fc. However, the IgY heavy chain contains a cysteine at position 347, which has no equivalent in either the IgE or the IgG heavy chains. These results suggest that this residue is capable of forming an interchain disulphide bond (Figure 3.4 iv), which allows the Cυ3-4 fragment to remain dimeric when denatured in SDS (Figure 3.4 iii).

3.5 Crystallisation of IgY-Fc

The purified IgY-Fc (Cυ3-4) was used to screen for crystallisation conditions using the hanging drop vapour diffusion method as described in chapter 2. Screens were examined after one week and crystals of various morphologies were observed under several conditions (Figure 3.5 i and Table 3.5 ii). An example of each crystal was stained with methylene blue to confirm that they were composed of protein.

Crystallisation conditions from hits on the initial screen were varied in order to optimise crystal growth. Crystals grew to the same size and morphology as noted in the initial screen under a range of pH, precipitant, buffer and/or additive concentrations, so several examples of each were wet mounted and tested for X-ray diffraction in-house. Others were cryo-cooled and tested at the Synchrotron Radiation Source (SRS), Daresbury, UK.

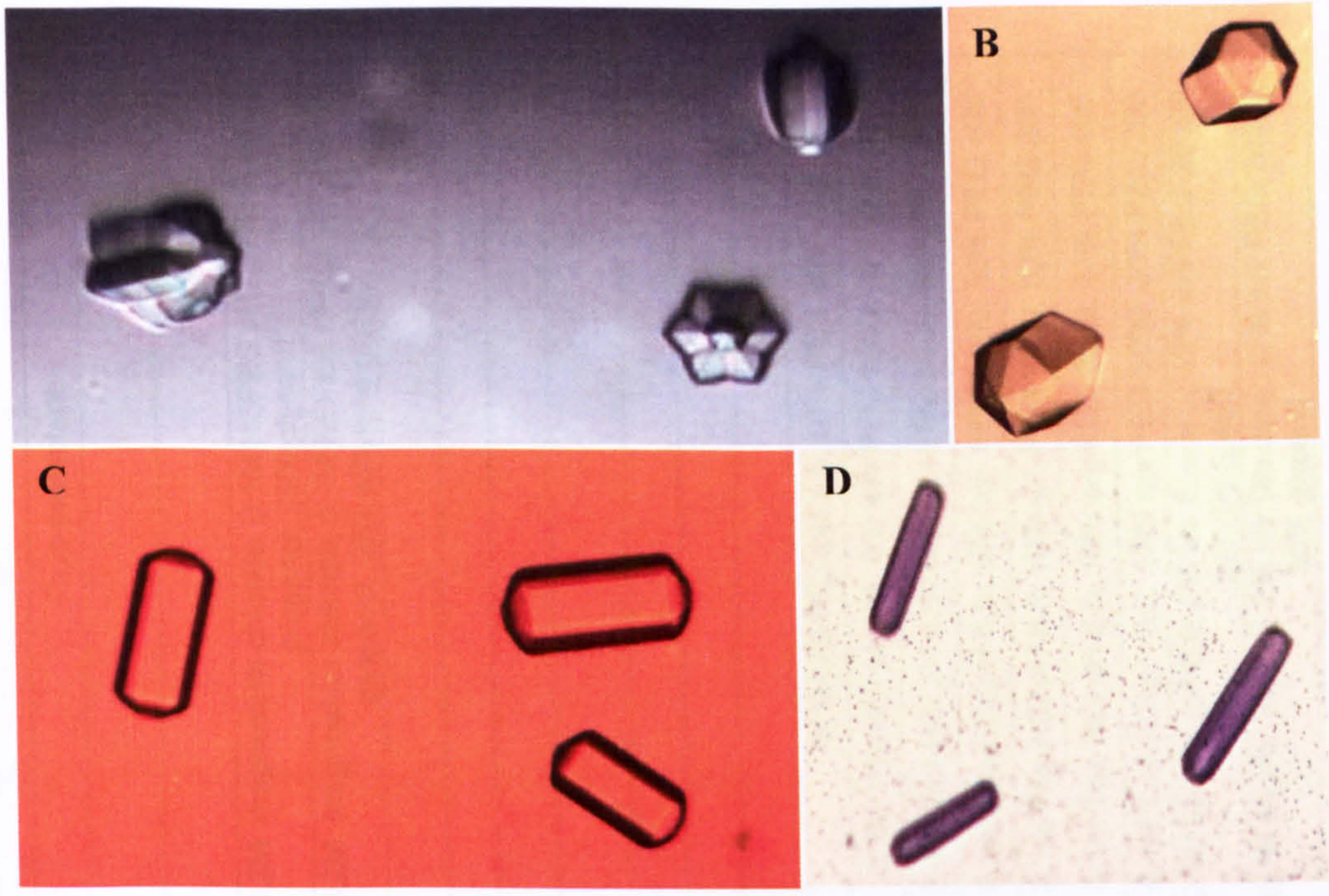


Figure 3.5 i
Crystals of IgY-Fc Cu3-4

Examples of each crystal morphology observed. Images B and C are of crystals of the same morphology taken from a different angle. Image D shows crystals stained with methylene blue. The longest dimensions observed for each morphology were: A = 100µm, B/C = 75µm, D = 150µm.

Crystal Type	Crystallisation Conditions	Cryo-protectant
A	0.2M sodium citrate tribasic dihydrate, 0.1M HEPES sodium salt pH 7.5, 20% v/v 2-propanol	
	0.2M sodium citrate tribasic dihydrate, 0.1M sodium cacodylate trihydrate pH 6.5, 30% v/v 2-propanol	
	0.2M magnesium chloride hexahydrate, 0.1M HEPES sodium salt pH 7.5, 30% v/v 2-propanol	0.2M magnesium chloride, 0.1M HEPES pH7.5, 35% v/v 2-propanol, 25% glycerol
		0.2M magnesium chloride, 0.1M HEPES pH7.5, 35% v/v 2-propanol, 25% ethylene glycol
	0.1M sodium citrate tribasic dihydrate pH 5.6, 1M ammonium dihydrogen phosphate	
	0.4M potassium sodium tartrate tetrahydrate	
	0.1M imidazole pH 6.5, 1M sodium acetate anhydrous	
	0.2M magnesium chloride hexahydrate, 0.1M HEPES sodium salt pH 7.6, 28% v/v 2-propanol	
B/C	0.4M potassium sodium tartrate terahydrate	
	0.1M Bicine pH 9.0, 2% v/v dioxane, 12% w/v PEG 20000	
	0.1M HEPES pH 7.5, 10% w/v PEG 6000, 5% v/v MPD	
	0.1M HEPES pH 7.5, 40% v/v PEG 200	0.1M HEPES pH 7.5, 50% PEG 200
	0.1M Tris-HCl pH 8.4, 6% w/v PEG 8000	0.1M Tris-HCl pH 8.5, 6% w/v PEG 8000, 30% glycerol
	0.1M Tris-HCl pH 8.6, 6% w/v PEG 8000	0.1M Tris-HCl pH 8.5, 10% w/v PEG 8000, 30% PEG 200
	0.1M Tris-HCl pH 8.5, 8% w/v PEG 8000	0.1M Tris-HCl pH 8.5, 10% w/v PEG 8000, 30% PEG 400
	0.1M Tris-HCl pH 8.6, 8% w/v PEG 8000	0.1M Tris-HCl pH 8.5, 10% w/v PEG 8000, 30% PEG 200
	0.1M Tris-HCl pH 8.5, 10% w/v PEG 8000	0.1M Tris-HCl pH 8.5, 10% w/v PEG 8000, 30% PEG 400
	0.1M Tris-HCl pH 8.6, 10% w/v PEG 8000	0.1M Tris-HCl pH 8.5, 10% w/v PEG 8000, 30% PEG 200
D	0.1M Bicine pH 8.9 2% v/v dioxane, 10% w/v PEG 20000	0.1M Bicine pH 8.9, 2% v/v Dioxane, 12% w/v PEG 20000, 30% PEG 400
		0.1M Bicine pH 8.9, 2% v/v Dioxane, 12% w/v PEG 20000, 30% glycerol

Table 3.5 ii (previous page)
Crystallisation and cryo-protectant conditions for IgY-Fc

The crystal type refers to the morphology detailed in figure 3.5 i. For those crystals that were cryo-cooled, protectants found to be suitable are shown. Acknowledgement to Dr. A. Davies & Dr. S. Fabiane for assistance in cryo-protectant screening

3.6 X-ray diffraction of IgY-Fc crystals

The wet-mounted crystals tested in-house diffracted to ~11 Å resolution and the cryo-cooled crystals tested at SRS (Daresbury, UK) to ~8 Å resolution and the diffraction patterns confirm that they were indeed protein. Diffraction images from one of the cryo-cooled crystals are shown in figures 3.6 i and 3.6 ii. Unfortunately, these resolutions were insufficient to provide useful structural data.

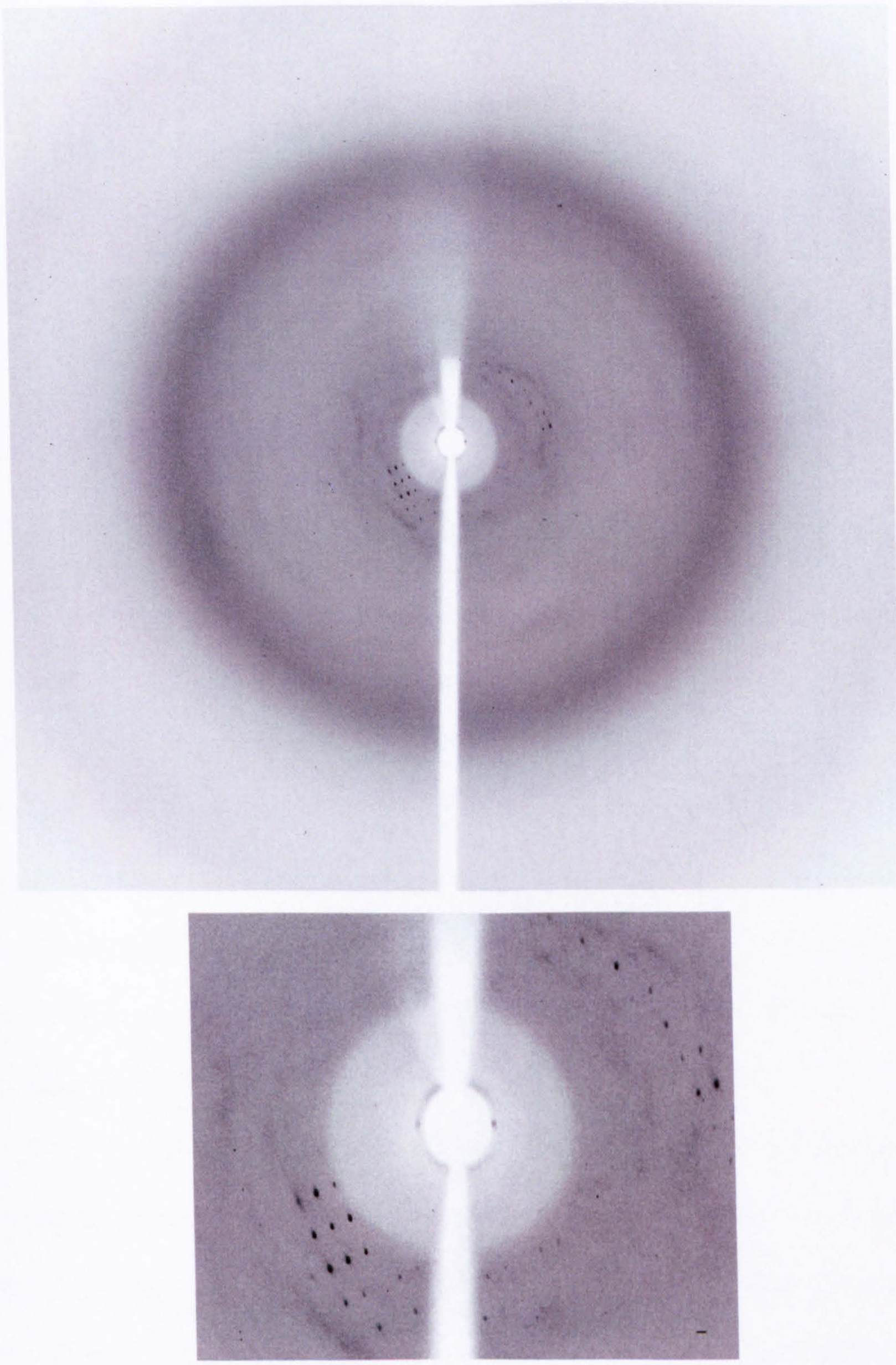


Figure 3.6 i
X-ray diffraction image of an IgY-Fc crystal to ~8Å resolution

Image was collected at beamline 10.1, Synchrotron Radiation Source (SRS), Daresbury, UK. Crystal to detector distance = 240mm, Exposure time = 60s, $\Delta\Phi = 1^{o}$. Data collection was performed by Dr. A. Davies and Dr. M. Holdom.

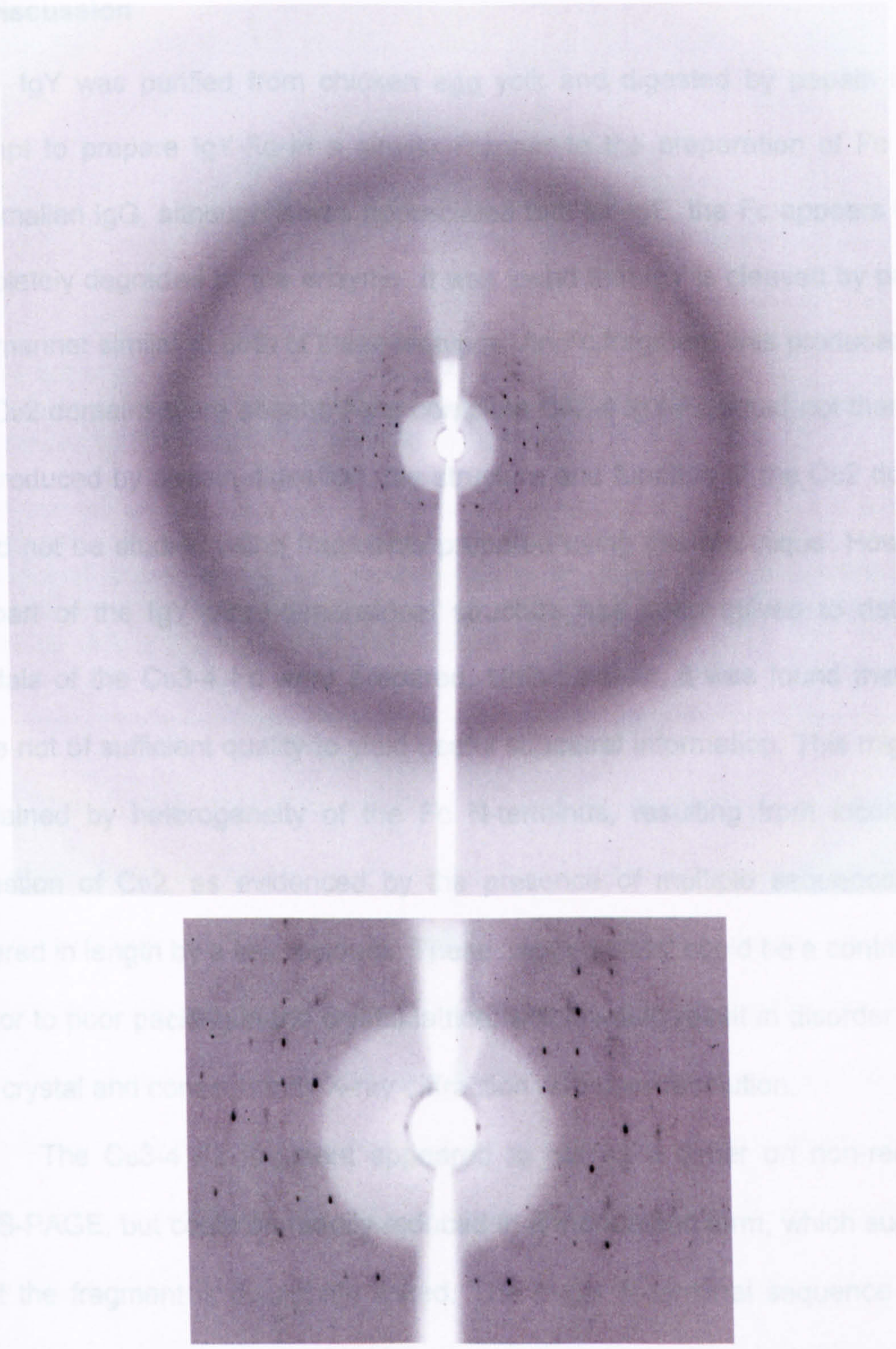


Figure 3.6 ii

X-ray diffraction image of crystals of IgY-Fc

Image was collected at beamline 10.1, Synchrotron Radiation Source (SRS), Daresbury, UK. Distance = 240mm, Exposure time = 120s, $\Delta\Phi = 1^{o}$. This image is from the same crystal as figure 3.6 i, but rotated through 90 °. Data collection was performed by Dr. A. Davies and Dr. M. Holdom.

3.7 Discussion

IgY was purified from chicken egg yolk and digested by papain in an attempt to prepare IgY-Fc in a similar manner to the preparation of Fc from mammalian IgG, although it was appreciated that for IgE, the Fc appears to be completely degraded by the enzyme. It was found that IgY is cleaved by papain in a manner similar to both of these isotypes. An Fc fragment was produced, but the Cυ2 domains were absent. As a complete Cυ2-4 IgY-Fc could not therefore be produced by papain digestion, the structure and function of the Cυ2 domain could not be studied using fragments prepared using this technique. However, no part of the IgY three-dimensional structure has been solved to date, so crystals of the Cυ3-4 Fc were prepared. Unfortunately, it was found that they were not of sufficient quality to yield useful structural information. This might be explained by heterogeneity of the Fc N-terminus, resulting from incomplete digestion of Cυ2, as evidenced by the presence of multiple sequences that differed in length by a few residues. These 'ragged ends' could be a contributing factor to poor packing in the crystal lattice, which would result in disorder within the crystal and consequently X-ray diffraction with poor resolution.

The Cυ3-4 Fc fragment appeared to run as a dimer on non-reducing SDS-PAGE, but could be readily reduced to a monomeric form, which suggests that the fragment is disulphide linked. The major N-terminal sequence of the dimeric band showed that of the three cysteines in the heavy chain sequence that could be involved in interchain bonds, the only one present was C347, the "extra" cysteine that has no equivalent in IgE (Figure 3.7 i).

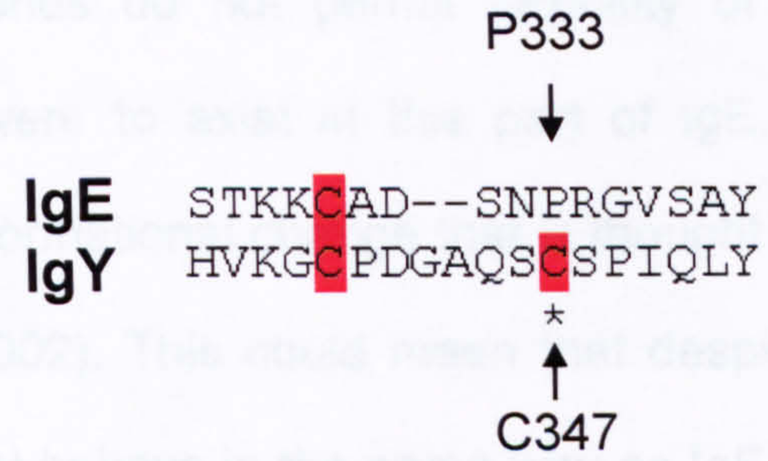


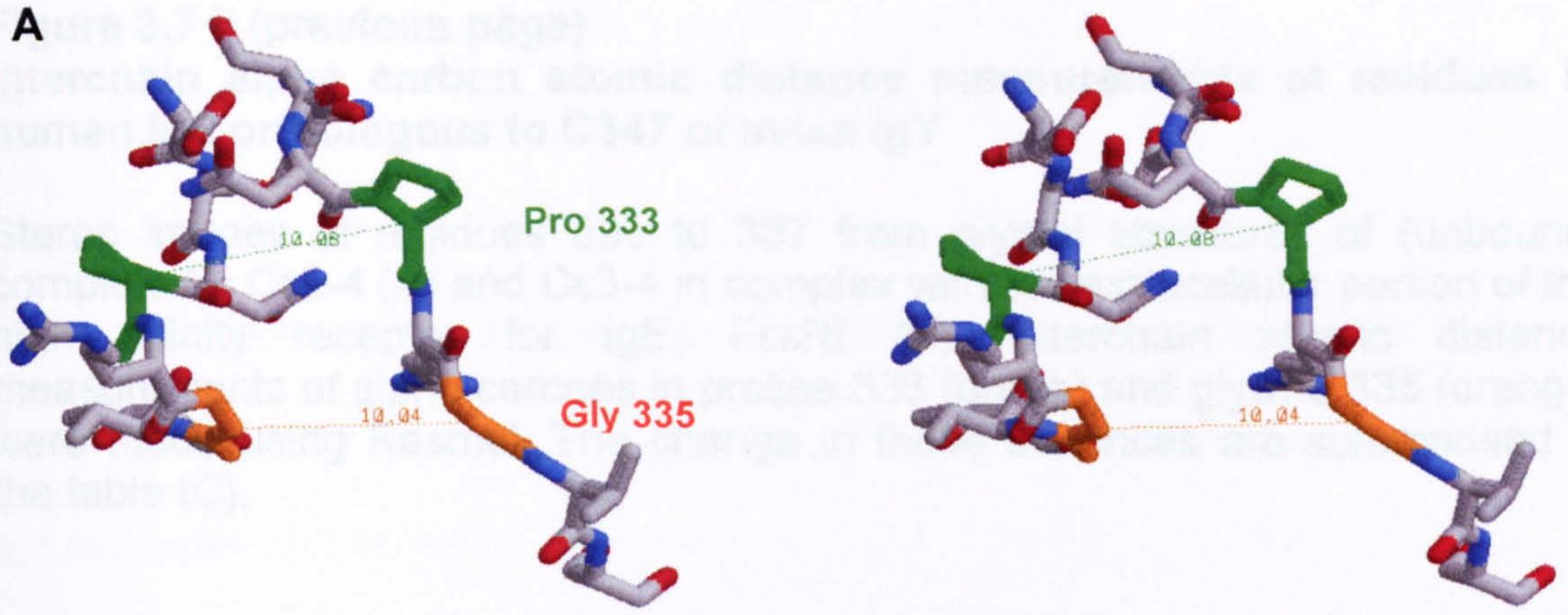
Figure 3.7 i
Alignment of human epsilon and chicken upsilon Ig heavy chain cDNA sequences at the CH2 / CH3 domain boundary

An alignment of the complete heavy chain constant region from IgE and IgY was made using ClustalW. The upsilon chain contains a cysteine residue (C347) that is not predicted to be involved in an interdomain disulphide bond. The epsilon chain lacks an equivalent cysteine, so IgY may have one more interchain disulphide bond than mammalian IgE.

The finding that this residue is capable of forming an interchain disulphide bond in the Cυ3-4 Fc could have important implications for the structure of IgY and its receptor interactions. An alignment of the upsilon and epsilon heavy chains suggests that C347 in IgY is replaced by P333 or G335 in IgE. The clustalW alignment software opens a two residue gap just before this region, so precisely which residue is a more likely substitute in IgE is unclear. Both of these residues are implicated in binding of IgE to its high affinity receptor, FcεRI (Garman *et al.*, 2000). In the "open" conformation of receptor-bound Cε3-4 (Garman *et al.*, 2000), and the "closed" conformation of unbound Cε2-4 (Wan *et al.*, 2002), the change in interchain atomic distances between the alpha carbons (Cα) of P333 and G335 are both approximately 7Å; from 10.1Å to 17.1Å for P333 and from 10.04Å to 17.4Å for G335 (Figure 3.7 ii). Cα - Cα distances between disulphide-bonded cysteines have been noted in crystal

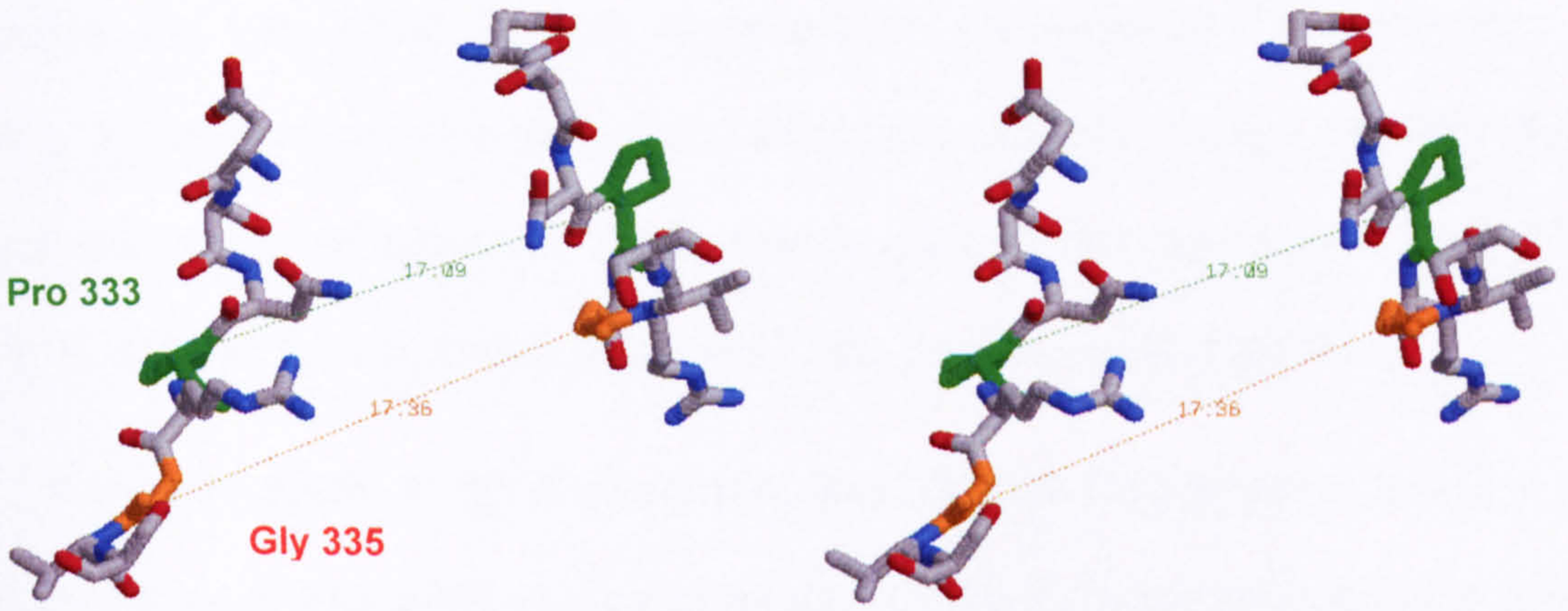
structures to range between 4.4Å and 9.3Å (Krovetz *et al.*, 1997), which would suggest that these bonds do not permit flexibility of more than 5Å. If an interchain disulphide were to exist in this part of IgE, constraints would be imposed upon the conformational change that is thought to occur upon receptor binding (Wan *et al.*, 2002). This could mean that despite numerous structural similarities, IgY may not behave in the same way as IgE upon receptor binding, which may affect the affinity and kinetics of the interaction.

A



Unbound (Cε2-4, Wan *et al.*, 2002)

B



Bound to receptor (Cε3-4 / FcεRIα complex, Garman *et al.*, 2000)

C

	Interchain Cα-Cα distance (Angstroms)	
	Proline 333	Glycine 335
In Cε2-4 structure (unbound)	10.1	10.04
In Cε3-4 structure (bound to receptor)	17.1	17.4
Change upon receptor binding	7.02	7.3

Figure 3.7 ii (previous page)
Interchain alpha carbon atomic distance measurements of residues in human IgE orthologous to C347 of avian IgY

Stereo images of residues 330 to 337 from crystal structures of (unbound) complete Fc Cε2-4 (A) and Cε3-4 in complex with the extracellular portion of the high affinity receptor for IgE, FcεRI (B). Interchain atomic distance measurements of alpha carbons in proline 333 (green) and glycine 335 (orange) were made using Rasmol. The change in these distances are summarised in the table (C).

Chapter 4

Design and construction of IgY-Fc mammalian expression vectors

4.1 Overview

In the previous chapter, it was shown that papain digestion could not be used to separate a fragment from IgY that contains all of the domains that make up the Fc; Cυ2, Cυ3 and Cυ4. This has since been confirmed by another group (Suzuki and Lee, 2004). To date, crystals of IgY-Fc prepared in this manner have not been reported to yield X-ray diffraction data of sufficient resolution for analysis (Kubo and Benedict, 1969). Poor quality crystals could be explained by the N-terminal heterogeneity observed in the papain cleaved IgY-Fc.

In order to obtain an IgY-Fc fragment that contains Cυ2 domains, which is essential for comparative studies with IgE-Fc (Cε2-4), a recombinant form must be produced. Through recombinant DNA technology, large quantities of protein can be produced and mutations can be made that allow heterogeneity to be minimised, both of which are essential if highly ordered crystals are to be grown.

Vectors encoding IgE-Fc fragments (Cε2-4, Cε3-4 and various mutants) have been constructed by members of our group and used to express secreted material from mammalian cells for crystallography and biophysical investigations (Henry *et al.*, 1997; Hunt *et al.*, 2005; McDonnell *et al.*, 2001; Shi *et al.*, 1997; Wan *et al.*, 2002; Young *et al.*, 1995). Production rates from stable transfectants of up to 33μg/10^6 cells/24hrs were achieved, such that upwards of

100 milligrams could be synthesised per litre of culture (R.J. Young, 1994, PhD Thesis). To produce sufficient quantities of recombinant IgY-Fc for similar studies, an IgE-Fc expression vector, pRY24, was adapted to express IgY-Fc, with as few changes as possible.

The design of the Cε2-4 and Cε3-4 IgE-Fc fragments produced in the above studies were used as guides for design of IgY-Fc fragments.

4.2 Selection of mutations

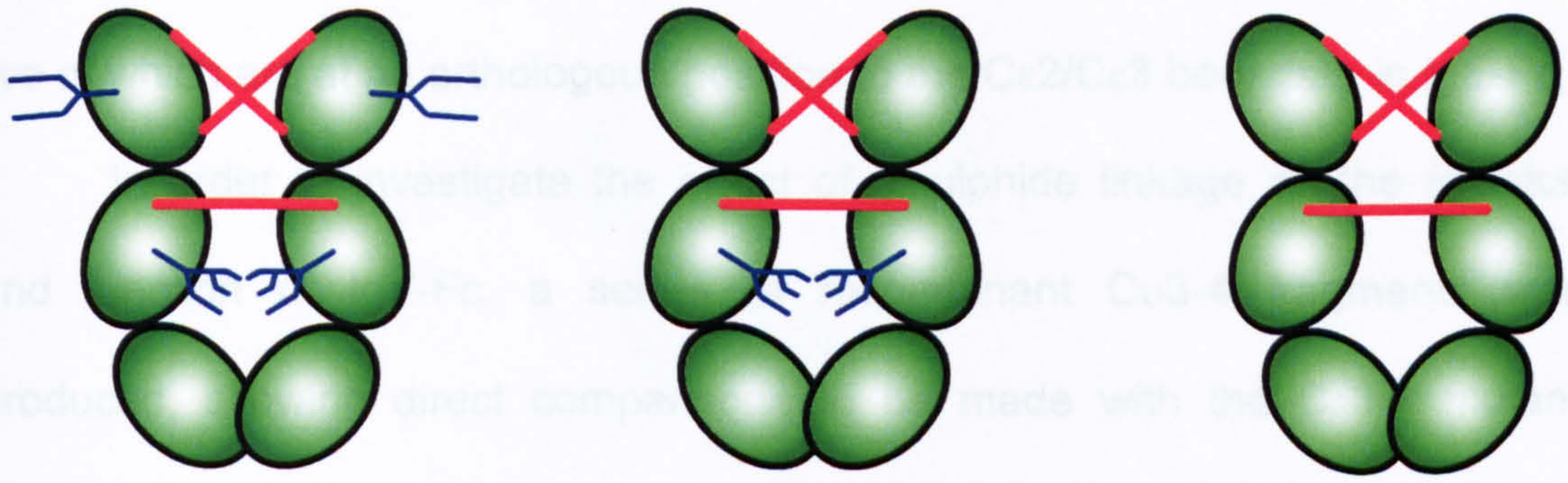
4.2.1 N-linked glycosylation

Carbohydrate moieties on human IgE and IgG play important roles in both structure and function. In IgG, the Fc has a horseshoe-like structure, with a core of complex-type oligosaccharides, linked to asparagine residues at position 297 in each Cγ2 domain. Glycosylation has been shown to be crucial for maintenance of normal IgG-Fc conformation and the ability to bind Fc receptors and complement (Kobata, 1990; Krapp *et al.*, 2003; Mimura *et al.*, 2000; Mimura *et al.*, 2001; Radaev and Sun, 2001; Tao and Morrison, 1989; Wright and Morrison, 1997; Woof, 1985, PhD Thesis).

The human IgE-Fc also contains complex-type glycosylation at N265 and N371. However, a third asparagine at position 394 is the orthologue of IgG's N297 and is linked to high-mannose oligosaccharide (Baenziger *et al.*, 1974a; Baenziger *et al.*, 1974b; Dorrington and Bennich, 1978; Wan *et al.*, 2002). Unlike IgG-Fc fragments, IgE-Fc fragments lacking carbohydrate are still able to bind the high affinity receptor for IgE, albeit with four-fold lower affinity (Basu *et al.*, 1993; Bjorklund *et al.*, 1999; Geha *et al.*, 1985; Helm *et al.*, 1988; Hunt *et al.*, 2005; Vercelli *et al.*, 1989).

The sequence of chicken IgY-Fc shows two potential N-glycosylation sites at N308 and N407, which are linked to complex and high-mannose type oligosaccharides, respectively (Suzuki and Lee, 2004). The location and type of glycosylation in IgY-Fc are more similar to those in IgE-Fc than IgG-Fc. N308 in chicken Cυ2 is orthologous to N265 in human Cε2 by virtue of it being the only glycosylation site in this domain, although its position is more similar to the Cε2 glycosylation sites of mouse, rat and opossum IgE. N407 in chicken Cυ3 appears to be orthologous to N394 in human Cε3, as the positions of the asparagines involved are well conserved between chicken, duck, human, rat and mouse Cυ3 and Cε3. In addition, the Cυ3- and Cε3-linked oligosaccharides in birds and mammals are all of the high-mannose type (Suzuki and Lee, 2004). The bent structure of Cε2-4 suggests that steric hindrance might prevent access to these sites by α-glucosidase II, an enzyme that is responsible for processing of N-linked carbohydrates (Wan *et al.*, 2002). In contrast, the structure of IgG-Fc imposes no such restriction (Zheng *et al.*, 1992), so glycosylation at N297 in Cγ2 becomes processed to complex type. The presence of high-mannose linked to Cυ3 might suggest that N407 is inaccessible in a similar manner to N394 in Cε3, which could imply a 3D structure that is more similar to IgE than IgG.

In order to investigate the effect of glycosylation on the structure and function of IgY-Fc, recombinant Cυ2-4 fragments were produced (i) with both sites intact ('wild type' or wt), (ii) asparagine 308 mutated to glutamine (N308Q) and (iii) asparagines 308 AND 407 mutated to glutamine (N308Q N407Q) (Figure 4.2.1).



wt **N308Q** **N308Q N407Q**

Figure 4.2.1
Schematic representation of recombinant IgY-Fc (Cυ2-4) fragments

Three 'complete' IgY-Fc (Cυ2-4) fragments were designed to be expressed in mammalian cells with varying glycosylation states: An unmodified form (wt) and two mutants that have one (N308Q) or both (N308Q N407Q) N-linked glycosylation sites removed. Sites were removed by mutation of the specified asparagine codon to encode glutamine instead. Putative interchain disulphide bonds are shown in red and oligosaccharides are shown in blue.

4.2.2 Interchain disulphide bridges

Recent studies by Hunt *et al.* (2005), using a series of Cε3-4 fragments, suggest that a non-native interchain disulphide bond in the IgE-Fc at the Cε2/Cε3 boundary is required to enable both Cε3 domains to bind to a single high affinity receptor for IgE, FcεRI, thereby increasing the affinity of binding and reducing the rate of dissociation from the receptor. The maintenance of proximity of the Cε3 domains in the Cε3-4 fragment prevents artefactual 1:2 receptor interaction stoichiometry (Hunt *et al.*, 2005).

The sequence of the upsilon heavy chain and the results of the previous chapter suggest that the IgY-Fc may possess three interchain disulphide bonds, two of which are at an orthologous position to the Cε2/Cε3 boundary in IgE-Fc.

In order to investigate the effect of disulphide linkage on the structure and function of IgY-Fc, a series of recombinant Cυ3-4 fragments were produced, allowing direct comparisons to be made with the Cε3-4 mutants designed and synthesised in earlier investigations. The Cυ3-4 mutants were designed to have (a) both cysteines present ("wild type" or wt), (b) cysteine 340 mutated to serine (C340S), (c) cysteine 347 mutated to serine (C347S) and (d) cysteines 340 AND 347 mutated to serines (C340S C347S) (Figure 4.2.2). Cυ2-4 fragments with disulphide bond mutations have not been produced in this investigation. Biophysical investigations using Cε3-4 have shown that this fragment retains receptor binding affinity that is comparable to Cε2-4 and whole IgE (Hunt *et al.*, 2005; Keown *et al.*, 1997; McDonnell *et al.*, 2001). These data suggest that the orthologous Cυ3-4 will also bind, allowing the role of Cys 347 to be investigated.

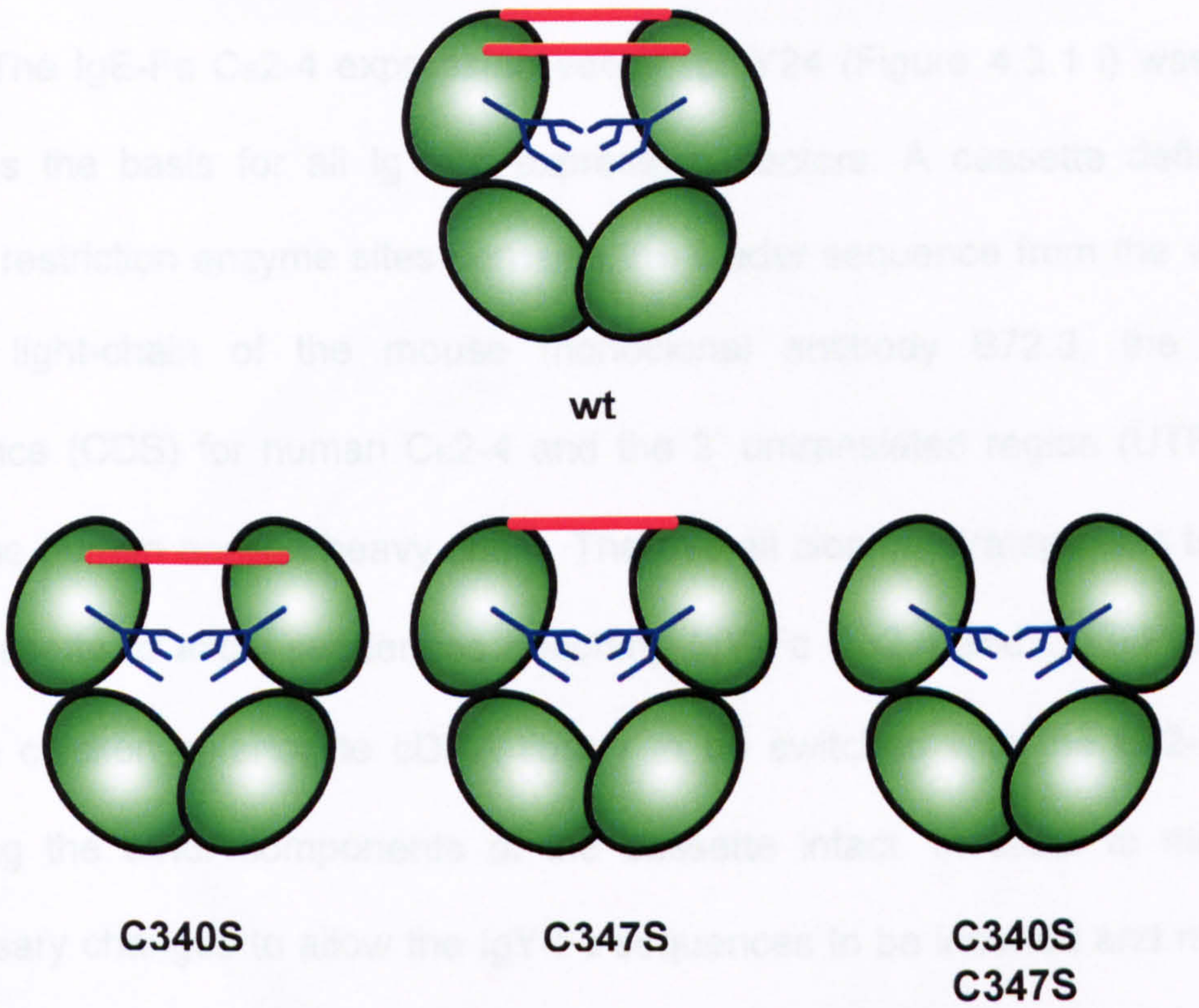


Figure 4.2.2
Schematic representation of recombinant IgY-Fc (Cυ3-4) mutants

Four IgY-Fc (Cυ2-4) sub-fragments were designed to be expressed in mammalian cells with various interchain disulphide bond configurations: an unmodified form (wt) and three mutants that contain cysteine residues in or one of two positions (C340S and C347S), or neither (C340S C347S). These residues may be involved in interchain disulphide bonds at the Cυ2/Cυ3 boundary. Where a disulfide was to be removed, the specified cysteine codon was mutated to encode serine instead. Putative interchain disulphide bonds are shown in red and oligosaccharides are shown in blue.

4.3 Construction of mutant IgY-Fc expression vectors

4.3.1 Overall strategy

The IgE-Fc Cε2-4 expression vector, pRY24 (Figure 4.3.1 i) was to be used as the basis for all IgY-Fc expression vectors. A cassette defined by HindIII restriction enzyme sites contains the leader sequence from the variable kappa light-chain of the mouse monoclonal antibody B72.3, the coding sequence (CDS) for human Cε2-4 and the 3' untranslated region (UTR), also from the human epsilon heavy chain. The overall cloning strategy was to adapt this cassette to allow sequences encoding IgY-Fc Cυ2-4 and Cυ3-4 amplified from a chicken splenocyte cDNA library to be switched with the Cε2-4 CDS, keeping the other components of the cassette intact. In order to make the necessary changes to allow the IgY-Fc sequences to be inserted and modified, as outlined in section 4.2, without inadvertent disruption to the rest of the pRY24 vector, the HindIII cassette was transferred into a small holding plasmid, pSP64T (Figure 4.3.1 ii). A summary of the cloning strategy is shown in figure 4.3.1 iii.

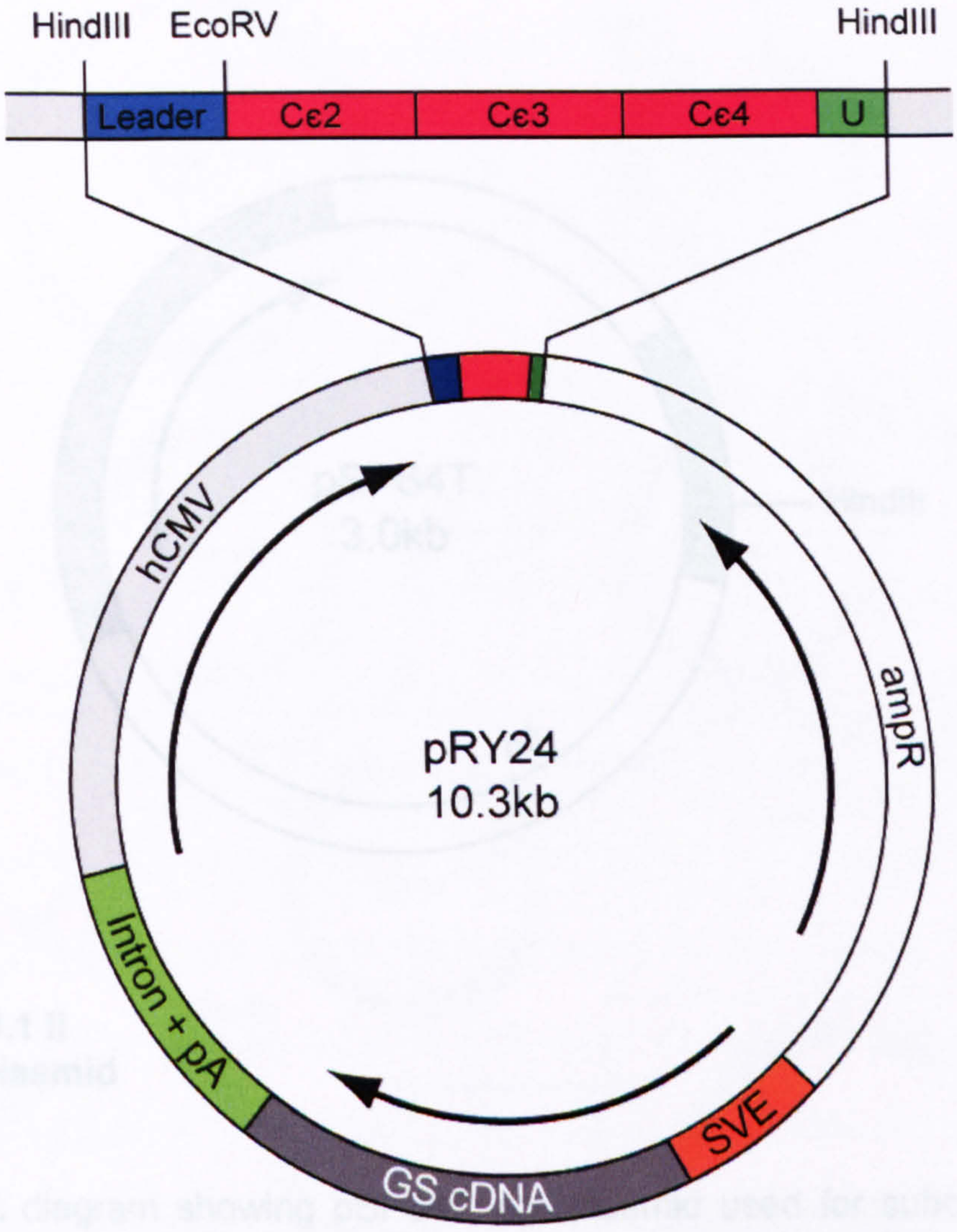


Figure 4.3.1 i
IgE-Fc expression vector for NS-0 cells, pRY24

Schematic diagram showing components of the mammalian expression vector pRY24, previously constructed in our group. pRY24 is based on pMRR018, a vector designed for stable transfection of NS-0 cells. pRY24 transfected NS-0s are highly efficient producers of secreted IgE-Fc (Cε2-4), whose expression is driven by the human cytomegalovirus immediate early promoter (hCMV). Expression of the glutamine synthetase (GS) gene (cDNA version) is driven by an SV40 early promoter (SVE) and allows selection of successfully transfected mammalian cells. The vector also contains an ampicillin resistance gene (ampR) in order to allow selection of transformed *E.coli* when cloning the plasmid. The IgE-Fc (Cε2-4) construct is contained within a HindIII fragment (top) that contains the coding sequence for domains Cε2, Cε3 and Cε4 bounded by a 3' untranslated region (labelled U) and a 5' leader sequence, which is derived from the B72.3 mouse monoclonal antibody variable kappa light chain. The leader sequence encodes a signal peptide that tags the Fc for transport to the endoplasmic reticulum for subsequent secretion.

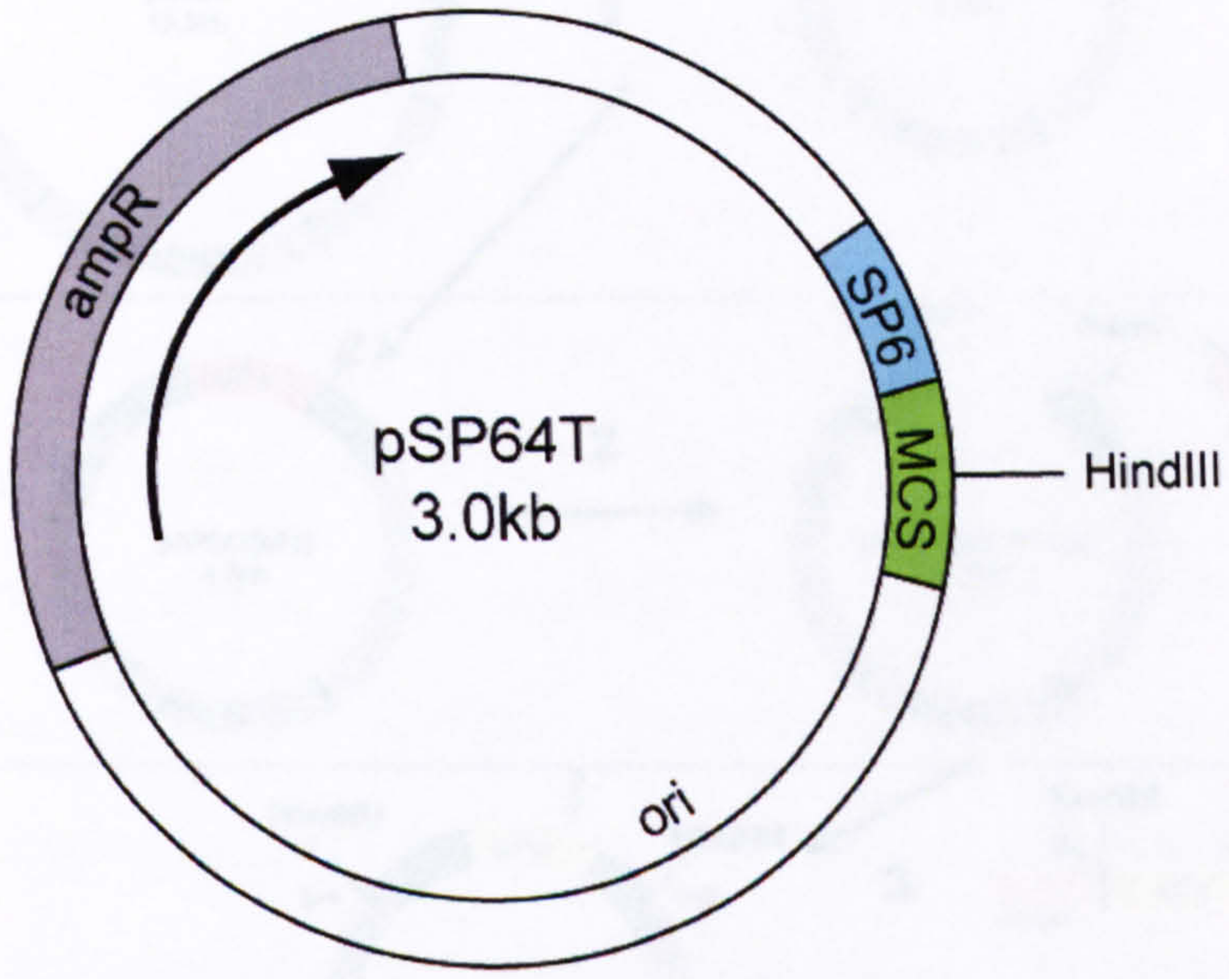


Figure 4.3.1 ii
pSP64T plasmid

Schematic diagram showing pSP64T, the plasmid used for subcloning of the IgE-Fc HindIII cassette from pRY24 into the plasmid's multiple cloning site (MCS), before insertion of either IgY-Fc (Cυ2-4) or IgY-Fc (Cυ3-4) cDNA. All site-directed mutagenesis on the IgY-Fc cassettes was performed in this plasmid rather than the final mammalian expression vector in order to avoid introduction of unwanted mutations by errors during PCR. The plasmid contains an ampicillin resistance gene (ampR) in order to allow selection of transformed *E.coli*.

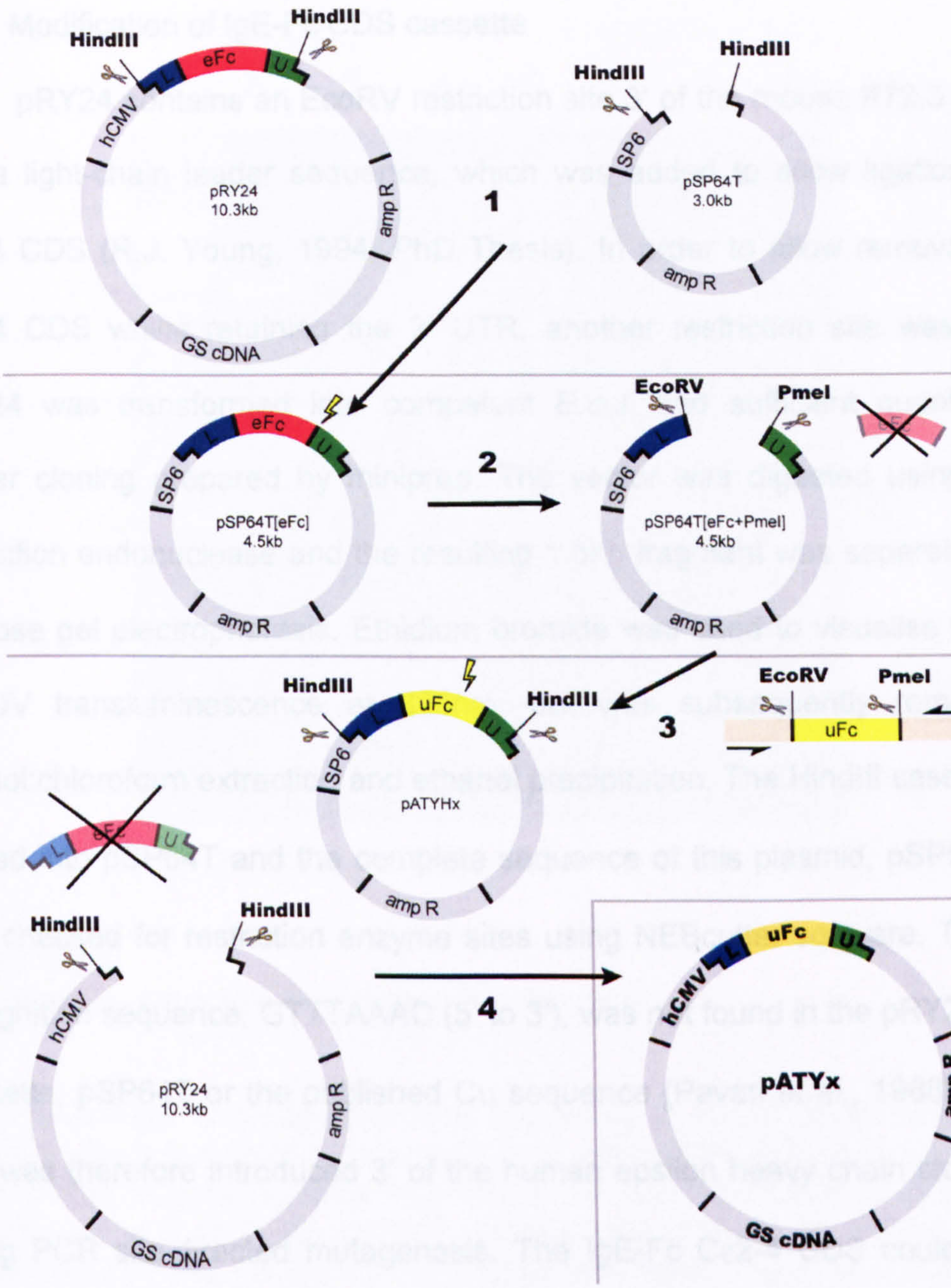


Figure 4.3.1 iii
Cloning strategy used to construct IgY-Fc expression vectors

HindIII restriction enzyme was used to excise a cassette consisting of the mouse monoclonal antibody B72.3 Vκ light chain leader sequence (L), the coding sequence (CDS) for human IgE-Fc (eFc) and the human epsilon heavy chain untranslated region (U) from the expression vector pRY24. The cassette was ligated into a small holding plasmid, pSP64T (**1**). Site-directed mutagenesis was used to introduce a PmeI recognition site (**2**) and a double digest with PmeI and EcoRV performed in order to remove the IgE-Fc CDS, which was then replaced with cDNA encoding chicken IgY-Fc (either Cυ2-4 or Cυ3-4) amplified by PCR from a chicken splenocyte cDNA library (**3**). Various mutations were made in different clones before the modified IgY-Fc cassettes were ligated in place of the IgE-Fc cassette in pRY24 to create a series of IgY-Fc expression vectors (**4**).

4.3.2 Modification of IgE-Fc CDS cassette

pRY24 contains an EcoRV restriction site 3' of the mouse B72.3 variable kappa light-chain leader sequence, which was added to allow ligation to the Cε2-4 CDS (R.J. Young, 1994, PhD Thesis). In order to allow removal of the Cε2-4 CDS whilst retaining the 3' UTR, another restriction site was added; pRY24 was transformed into competent *E.coli* and sufficient quantities for further cloning prepared by miniprep. The vector was digested using HindIII restriction endonuclease and the resulting 1.5kb fragment was separated using agarose gel electrophoresis. Ethidium bromide was used to visualise the DNA by UV transluminescence at 325nm, but was subsequently removed by phenol:chloroform extraction and ethanol precipitation. The HindIII cassette was ligated into pSP64T and the complete sequence of this plasmid, pSP64T[εFc], was checked for restriction enzyme sites using NEBcutter software. The PmeI recognition sequence, GTTTAAAC (5' to 3'), was not found in the pRY24 HindIII cassette, pSP64T or the published Cυ sequence (Pavari *et al.*, 1988). A PmeI site was therefore introduced 3' of the human epsilon heavy chain stop codon, using PCR site-directed mutagenesis. The IgE-Fc Cε2-4 CDS could then be removed from this plasmid, by performing a double digest with EcoRV and PmeI (Figure 4.3.2), followed by gel purification. 5' phosphates were removed using shrimp alkaline phosphatase in order to prevent formation of 'empty' plasmids in subsequent ligation reactions.

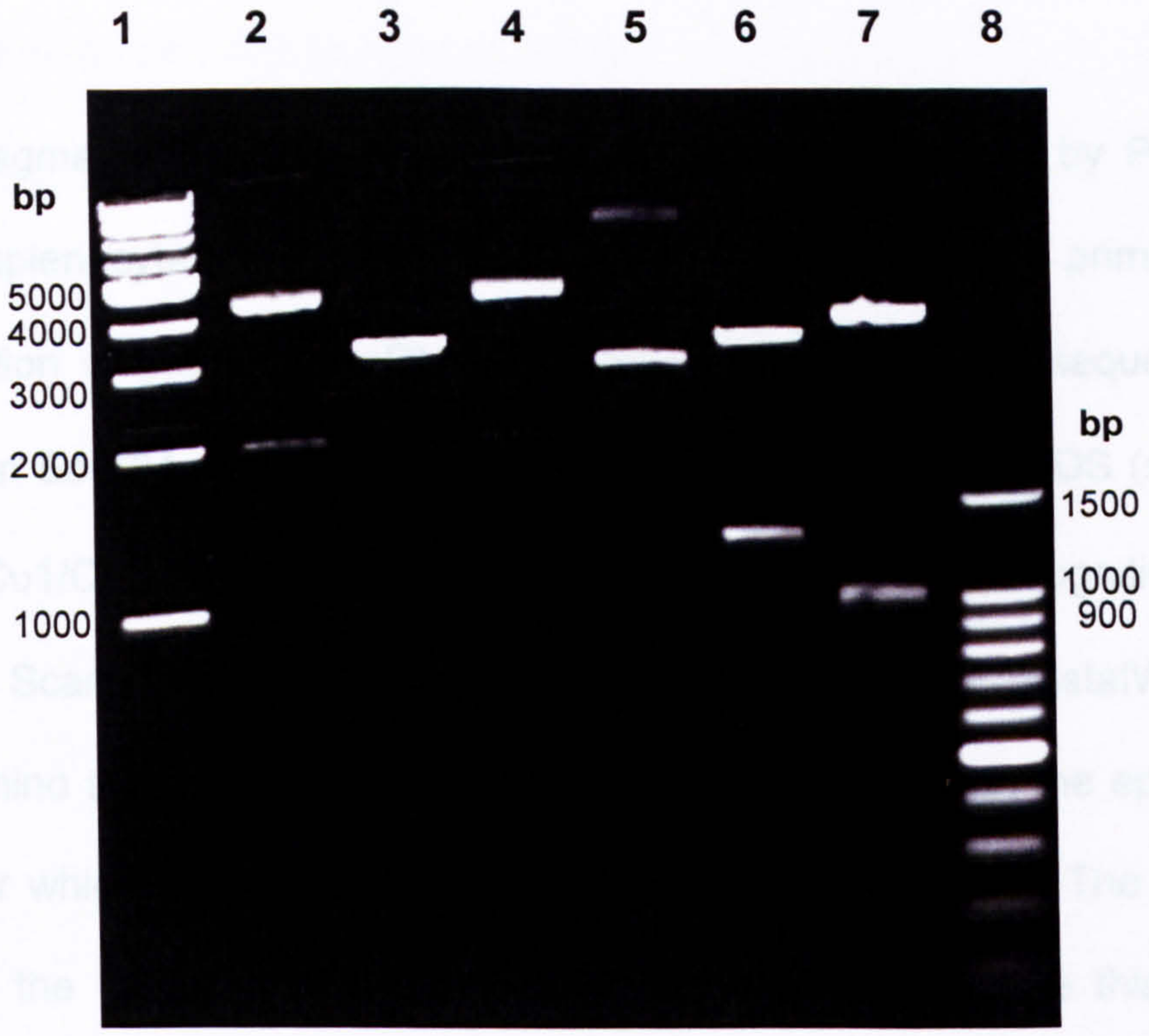


Figure 4.3.2
Preparation of the IgY-Fc expression cassette

1% agarose gel showing restriction fragments or uncut plasmids at various stages in the insertion of a PmeI restriction site into the IgE-Fc HindIII cassette from pRY24 in order to allow removal of the εFc CDS whilst retaining the 5' leader and the 3' UTR. (2) pSP64T plasmid without insert. (3) pSP64T cut with HindIII. (4) pSP64T incubated with EcoRV and PmeI. These enzymes have no restriction sites in the 'empty' plasmid. (5) pSP64T[εFc]. (6) pSP64T[εFc] cut with HindIII. (7) pSP64T[εFc+PmeI] cut with EcoRV and PmeI. Molecular weight markers: (1) 1kb ladder (8) 100bp ladder.

4.3.3 Amplification of Cυ2-4 and Cυ3-4 cDNA and creation of mutagenic cassettes

Fragments encoding Cυ2-4 and Cυ3-4 were amplified by PCR from a chicken splenocyte cDNA library (Figure 4.3.3 i). The forward primer used for amplification of the Cυ2-4 cDNA was designed so that the sequence would include an EcoRV site immediately 5' of the start of the Cυ2 CDS (see chapter 2). The Cυ1/Cυ2 junction was predicted by protein pattern recognition software (InterPro Scan, EMBL-EBI and ScanProsite, ExPASy) and ClustalW alignment of the amino acid sequences of the upsilon heavy chain with the epsilon heavy chain, for which the domain junctions have been established. The primer also changes the cysteine at position 232 to an alanine because this residue is orthologous to C225 in human Cε2, which forms a bridge to Cε1 (after Young *et al.*, 1995).

The Cυ2/Cυ3 junction was predicted as above, but the forward primer used to amplify Cυ3-4 cDNA was designed to begin the fragment at the penultimate residue in Cυ2, in order to include cysteine 340, which may form an interchain disulphide bond and help to give a less artefactual representation of the IgY-Fc Cυ3-4 fragment (after Shi *et al.*, 1997). The modifications made to the IgY-Fc cDNA ends are detailed in figure 4.3.3 ii.

The amplified cDNAs were digested with EcoRV and PmeI, then gel purified to prepare them for ligation into the cassette prepared in 4.3.2; successfully ligated plasmids were cloned by transformation of competent *E.coli* and selection on ampicillin LB-agar. Colonies containing plasmids with correctly oriented insert were selected by PCR screening and sequencing (data not

shown). The two resulting constructs were named pATHY24 and pATYH34 (summarised as pATYHx in figure 4.3.1 iii).

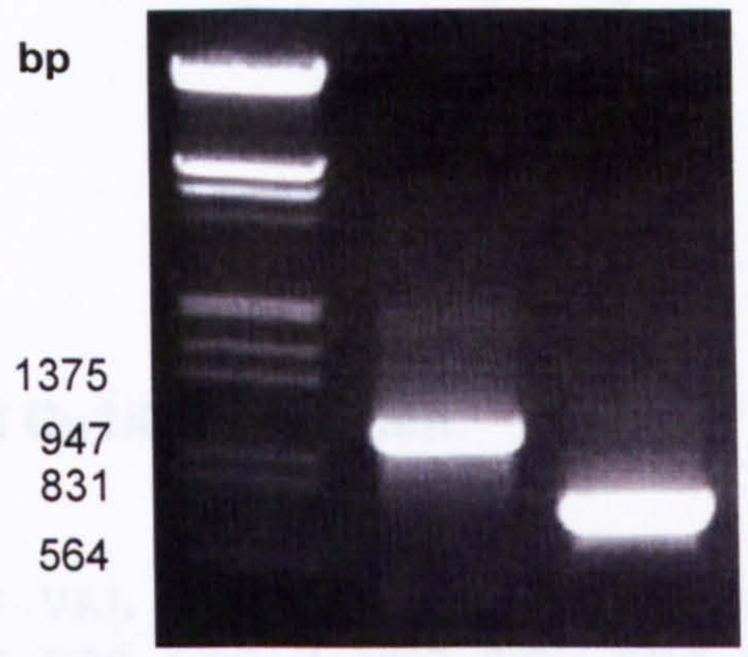


Figure 4.3.3 i
Amplification of IgY-Fc cDNA

cDNAs encoding (2) Cυ2-4 and (3) Cυ3-4 bounded by EcoRV and PmeI restriction sites were amplified by PCR using a chicken splenocyte cDNA library as template. Lane 1 contains λ / EcoRI / HindIII molecular weight marker.

A

```
5'
ATG AGT GTG CCC ACT CAG GTC CTG GGG TTG CTG CTG CTG
TAC TCA CAC GGG TGA GTC CAG GAC CCC AAC GAC GAC GAC
 M   S   V   P   T   Q   V   L   G   L   L   L   L

                                        EcoRV
TGG CTT ACA GAT GCC AGA TGT GAT...
ACC GAA TGT CTA CGG TCT ACA CTA...
 W   L   T   D   A   R   C   D
```

B

1. Predicted Cυ1/Cυ2 junction:

```
5'                          231
...LYS GLU VAL GLN / VAL CYS ARG VAL ASP...
...AAG GAG GTG CAG / GTC TGC CGG GTA GAT...
```

2. Leader sequence-adapter/Cυ2 junction:

```
5'                  ↓                V232A
MET...CYS ASP ILE / VAL ALA ARG VAL ASP...
ATG...TGT GAT ATC / GTC GCC CGG GTA GAT...
          EcoRV
```

3. Predicted Cυ2/Cυ3 junction:

```
5'          340       342                      347
...LYS GLY CYS PRO / ASP GLY ALA GLN SER CYS...
...AAG GGA TGC CCG / GAC GGC GCT CAG AGC TGC...
```

4. Leader sequence-adapter/Cυ3 junction:

```
5'          ↓           340
MET...CYS ASP ILE / CYS PRO ASP GLY ALA...
ATG...TGT GAT ATC / TGC CCG GAC GGC GCT...
          EcoRV
```

C

1. Natural Cε4/stop-UTR:

5'

...LYS SER ARG *Ter*

...AAA TCC CGG TAA ATG ACG TAC TCC TGC CTC CCT...

2. Cυ4/stop-adapter-UTR:

5'

...GLN ALA GLY LYS *Ter*

...CAG GCT GGT AAA TAA **GTT TAA ACC** TGC CTC CCT...

PmeI

Figure 4.3.3 ii (above and previous page)
Adaptation of the chicken IgY-Fc sequences for secretion

(A) The region encoding a signal peptide in the mouse variable kappa light chain leader sequence from monoclonal antibody B72.3, to which the IgY-Fc sequences were ligated. The leader has been mutated to introduce an EcoRV site.
(B) The residues at the inter domain junctions of the upsilon heavy chain (1,3) were predicted by motif identification software and alignment with the known boundaries of the epsilon heavy chain of human IgE. The presence of the EcoRV site and the location of the signal peptide processing site (marked with an arrow) means that all constructs have an aspartic acid residue and an isoleucine residue at the N-terminus. The Cυ2-4 cDNA was amplified with primers that change the cysteine at position 232 to an alanine (2). The recombinant Cε3-4 fragment used in previous studies (Shi *et al.*, 1997) did not begin precisely at the Cε3 junction; the last two amino acids in Cε2 were included so as to allow an interchain disulphide to form. IgY contains two cysteines in this locale (C340 and C347) and in order to be able to investigate their effect on the Cυ3-4 fragment, both were included by mimicking the Cε3-4 and beginning the construct at the penultimate residue of Cυ2 (4).
(C) The 3' untranslated region (UTR) of the epsilon heavy chain (1) was spliced to IgY-Fc cDNA by insertion of a PmeI restriction site (2).

4.3.4 Mutation of IgY-Fc Cυ2-4 and Cυ3-4 cassettes

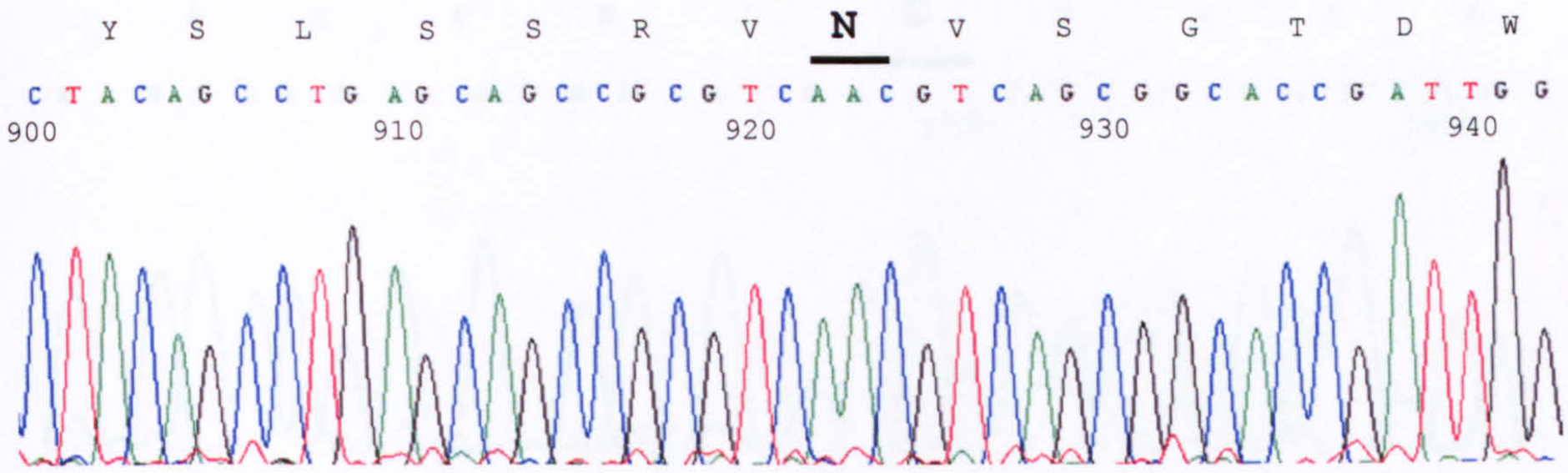
Site-directed mutagenesis was used to make the mutations described in section 4.2. The mutagenic primers (see chapter 2) were designed to alter the encoded amino acid, with as few changes to the nucleotide sequence as possible. The changes are summarised in table 4.3.4 i and sequencing chromatograms are shown in figure 4.3.4 ii.

Template Plasmid	Mutation	New Plasmid Name
pATYH24	none	pATYH24[wt]
pATYH24	N308Q	pATYH24[N308Q]
pATYH24[N308Q]	N407Q	pATYH24[N308Q N407Q]
pATYH34	none	pATYH34[wt]
pATYH34	C340S	pATYH34[C340S]
pATYH34	C347S	pATYH34[C347S]
pATYH34[C340S]	C347S	pATYH34[C340S C347S]

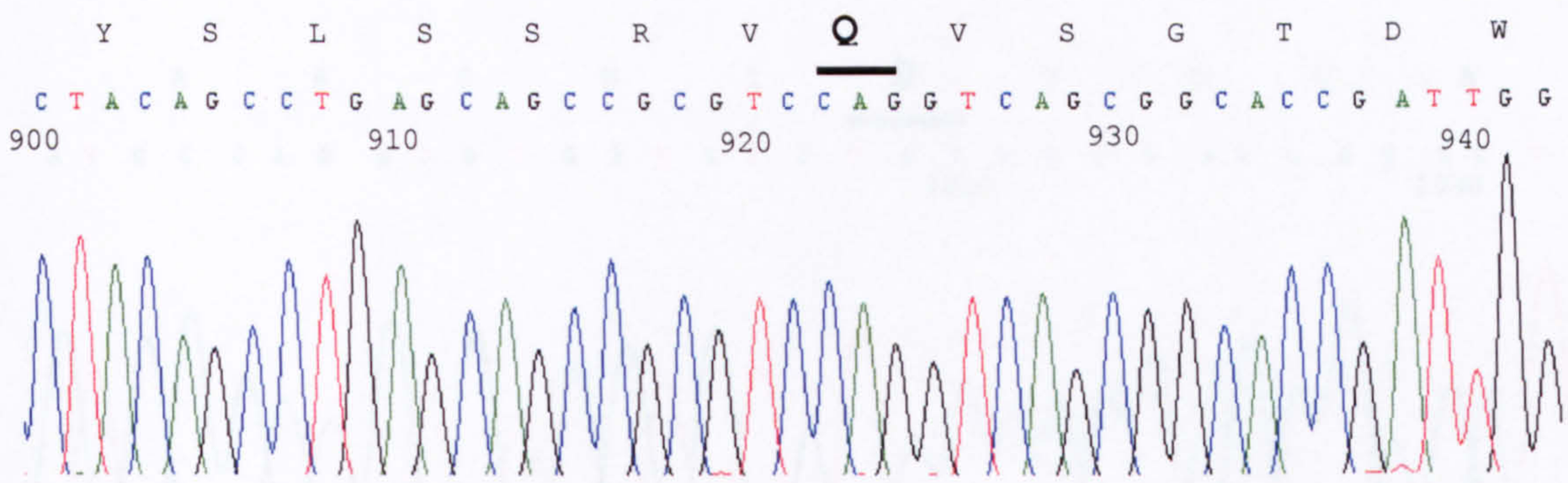
Table 4.3.4 i
Summary of mutations made in Cυ2-4 and Cυ3-4 coding sequences

As each mutation was made, the cassettes were sequenced (Figure 4.3.4 ii). The mutant and original 'wild type' sequences were found to be identical except for the intended changes.

1A

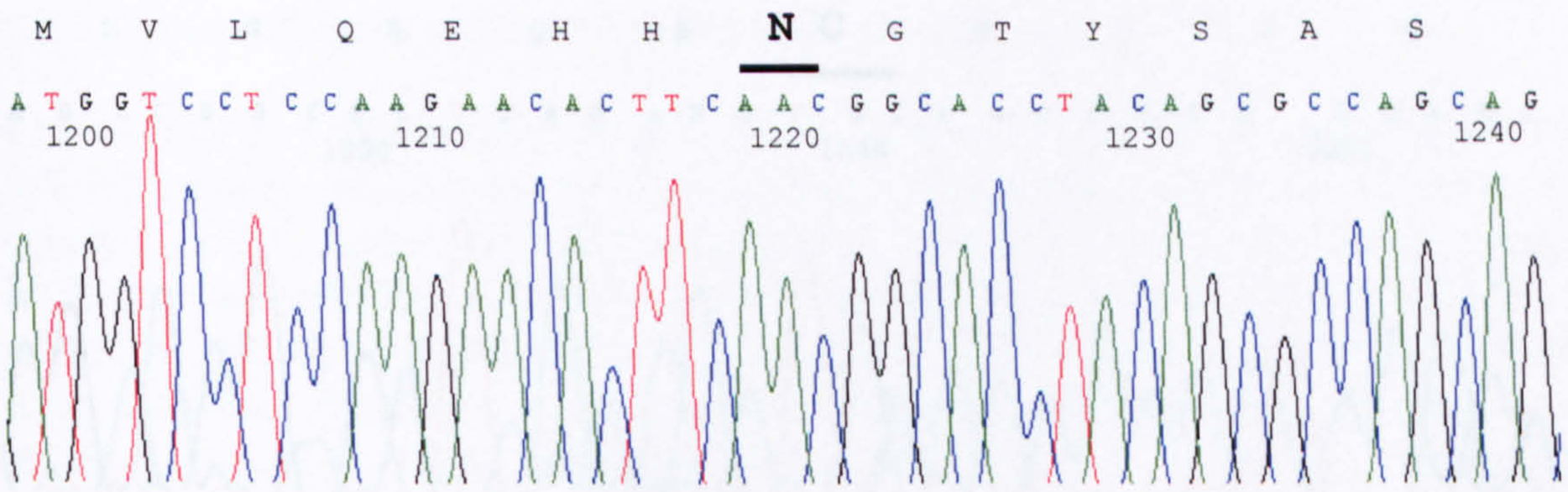


Cυ2-4 wild type

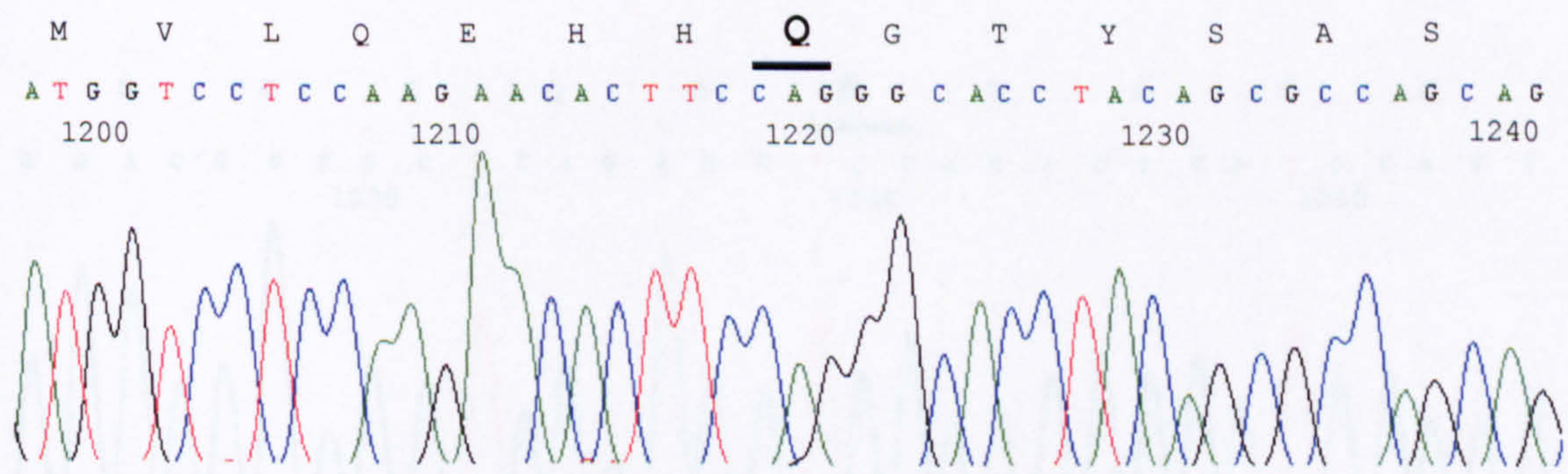


N308Q mutant

1B

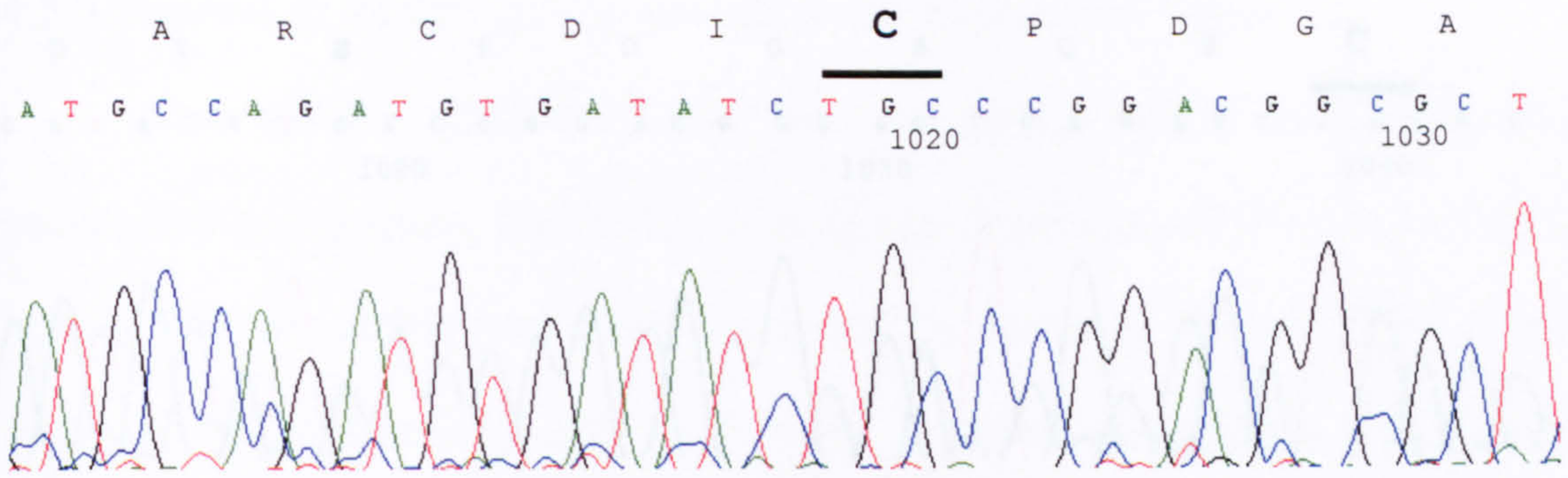


N308Q mutant



N308Q N407Q mutant

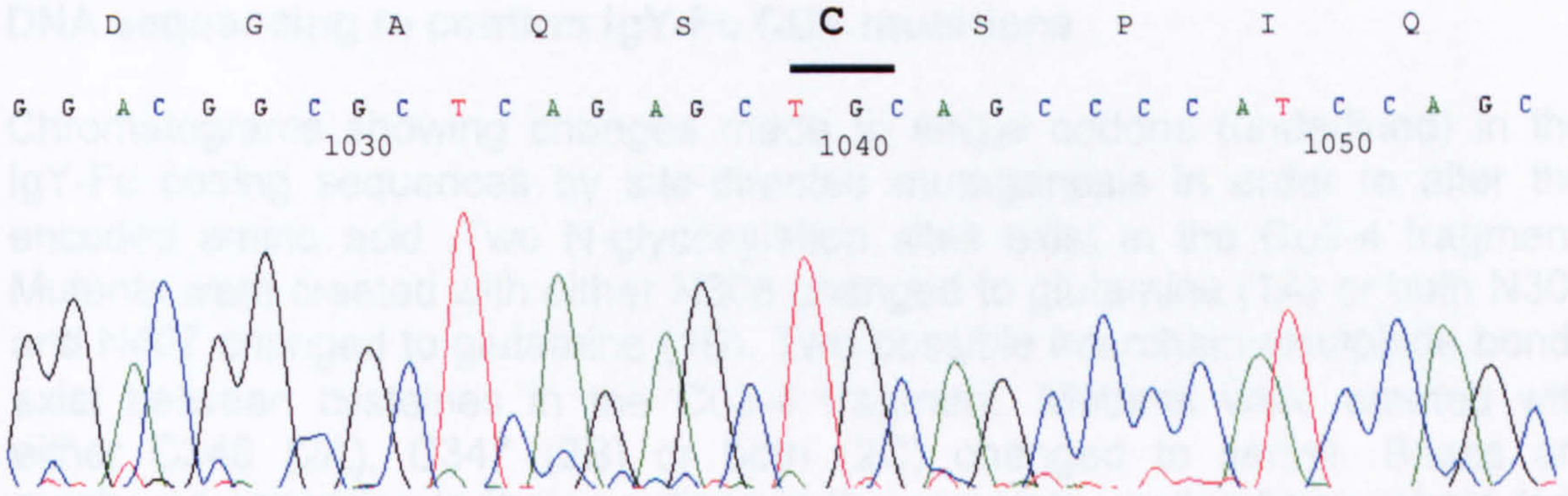
2A



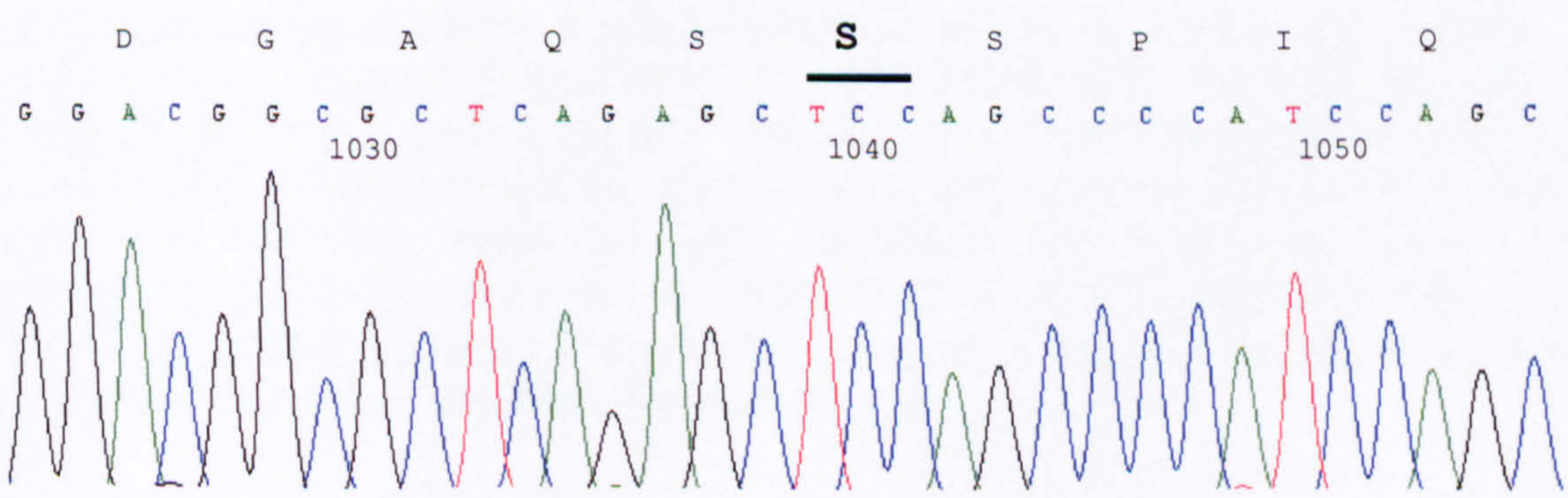
Cυ3-4 wild type

C340S

2B

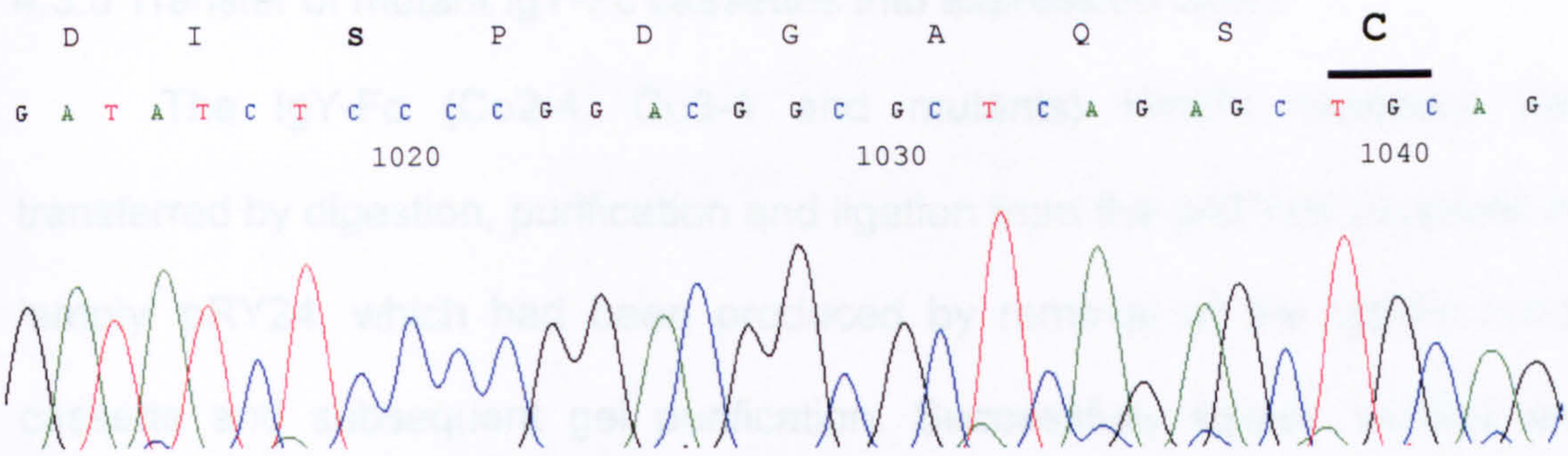


Cυ3-4 wild type

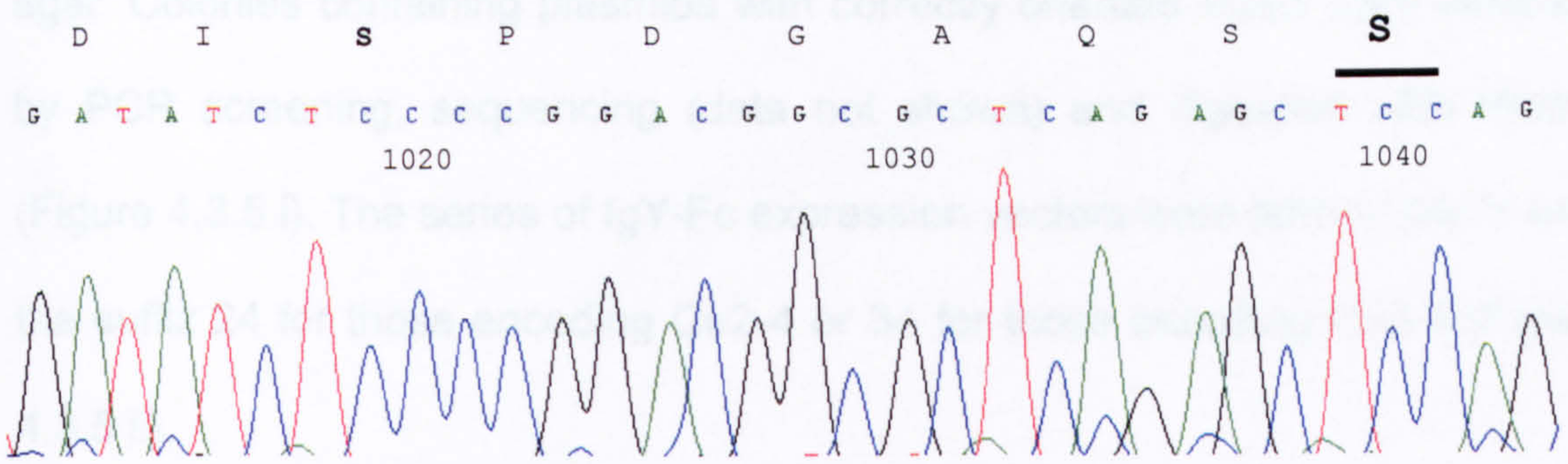


C347S mutant

2C



C340S mutant



C340S C347S mutant

Figure 4.3.4 ii (above and previous two pages)
DNA sequencing to confirm IgY-Fc CDS mutations

Chromatograms showing changes made to single codons (underlined) in the IgY-Fc coding sequences by site-directed mutagenesis in order to alter the encoded amino acid. Two N-glycosylation sites exist in the Cυ2-4 fragment. Mutants were created with either N308 changed to glutamine (1A) or both N308 and N407 changed to glutamine (1B). Two possible interchain disulphide bonds exist between cysteines in the Cυ3-4 fragment. Mutants were created with either C340 (2A), C347 (2B) or both (2C) changed to serine. Bases are numbered according to their positions in the complete upsilon heavy chain (see appendix 1).

4.3.5 Transfer of mutant IgY-Fc cassettes into expression vector

The IgY-Fc (Cυ2-4, Cυ3-4 and mutants) HindIII cassettes were transferred by digestion, purification and ligation from the pATYHx plasmids into 'empty' pRY24, which had been produced by removal of the IgE-Fc HindIII cassette and subsequent gel purification. Successfully ligated vectors were cloned by transformation of competent *E.coli* and selection on ampicillin LB-agar. Colonies containing plasmids with correctly oriented insert were selected by PCR screening, sequencing (data not shown) and digestion with HindIII (Figure 4.3.5 i). The series of IgY-Fc expression vectors were termed pATY with the suffix 24 for those encoding Cυ2-4 or 34 for those encoding Cυ3-4 (Figure 4.3.5 ii).

Figure 4.3.5 i
Confirmation of ligation of IgY-Fc cassettes into expression vector by digestion with HindIII

1% agarose gel showing digestion products of pATYx vectors with HindIII; (1) pATY24[wt]; (2) pATY24[N308Q]; (3) pATY24[N308Q / N407Q]; (4) pATY34 [wt]; (5) pATY34 [C340S]; (6) pATY34[C347S]; (7) pATY34 [C340S / C347S]. A 1kb ladder molecular weight marker was run concurrently (M). All vectors produce only two fragments upon digestion; the pRY expression vector backbone (~8.8kb) and either the Leader-Cυ2-4-εUTR (1.3kb) or Leader-Cυ3-4-εUTR (0.99kb) cassette. The mutations made in the IgY-Fc sequences were substitutions, so do not affect the cassette size.

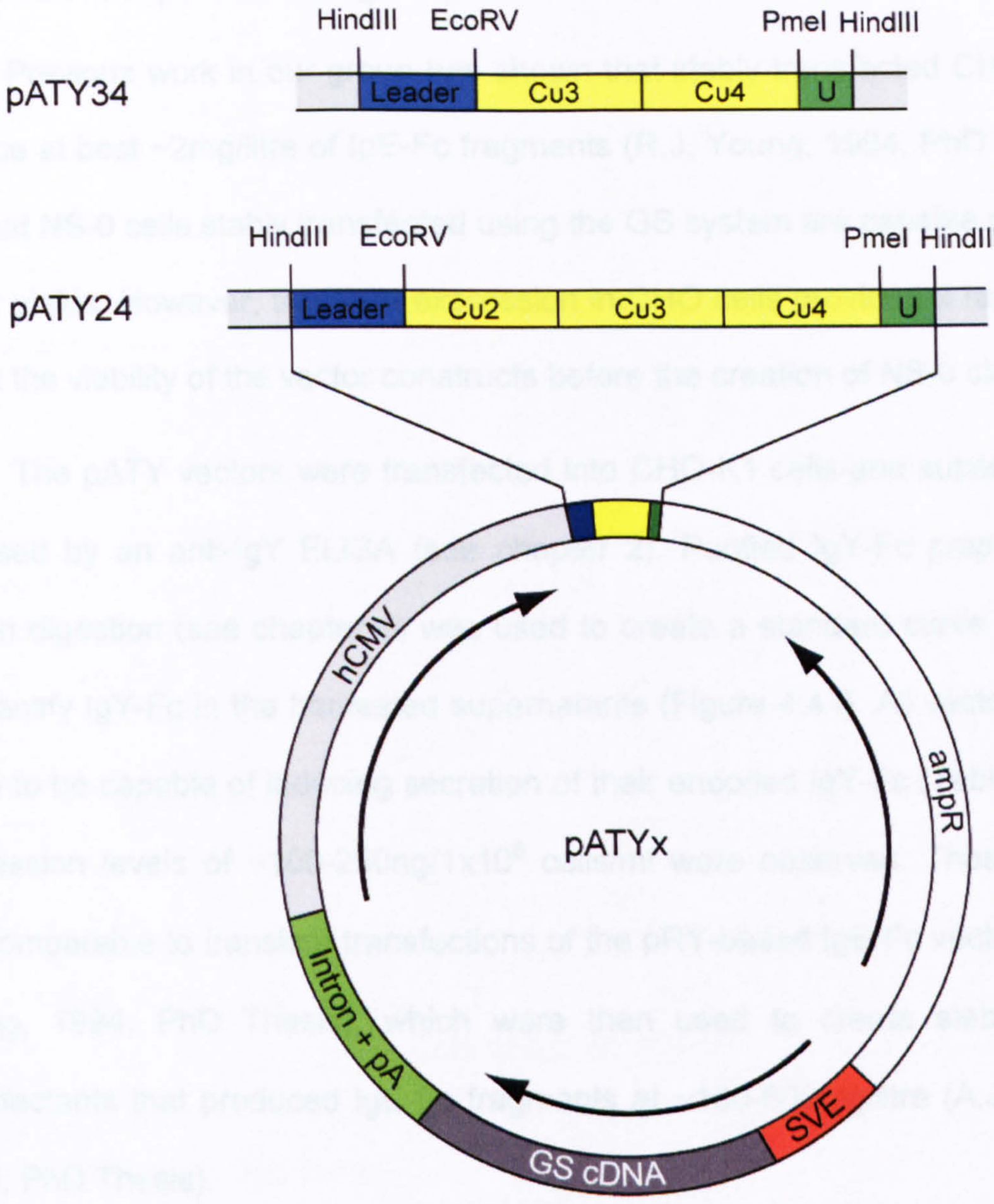


Figure 4.3.5 ii
IgY-Fc expression vector(s) for NS-0 cells, pATY24 & pATY34

The archetypal Cυ2-4 (pATY24) and Cυ3-4 (pATY34) expression vectors differ only in how much of the IgY-Fc coding sequence is present. These vectors and a series of mutants (not shown) are based on the highly productive IgE-Fc expression vector pRY24 (Young *et al.*, 1995). Expression of a construct of the mouse B72.3 monoclonal antibody variable kappa light chain 5' leader sequence, IgY-Fc CDS and the epsilon heavy chain 3' untranslated region (U) is driven by a human cytomegalovirus (hCMV) promoter. Expression of the glutamine synthetase (GS) gene (cDNA version) is driven by an SV40 early promoter (SVE) and allows selection of successfully transfected mammalian cells. The vector also contains an ampicillin resistance gene (ampR) in order to allow selection of transformed *E.coli* when cloning the plasmid.

4.4 Transient expression of IgY-Fcs in CHO cells

Previous work in our group has shown that stably transfected CHO cells produce at best ~2mg/litre of IgE-Fc fragments (R.J. Young, 1994, PhD Thesis) and that NS-0 cells stably transfected using the GS system are capable of much higher yields. However, transient expression in CHO cells provided a rapid way to test the viability of the vector constructs before the creation of NS-0 clones.

The pATY vectors were transfected into CHO-K1 cells and supernatants analysed by an anti-IgY ELISA (see chapter 2). Purified IgY-Fc prepared by papain digestion (see chapter 3) was used to create a standard curve in order to quantify IgY-Fc in the harvested supernatants (Figure 4.4 i). All vectors were found to be capable of inducing secretion of their encoded IgY-Fc (Table 4.4 ii). Expression levels of ~100-250ng/1x10^6 cells/ml were observed. These levels are comparable to transient transfections of the pRY-based IgE-Fc vectors (R.J. Young, 1994, PhD Thesis), which were then used to create stable NS-0 transfectants that produced IgE-Fc fragments at ~100-600mg/litre (A.J. Henry, 1996, PhD Thesis).

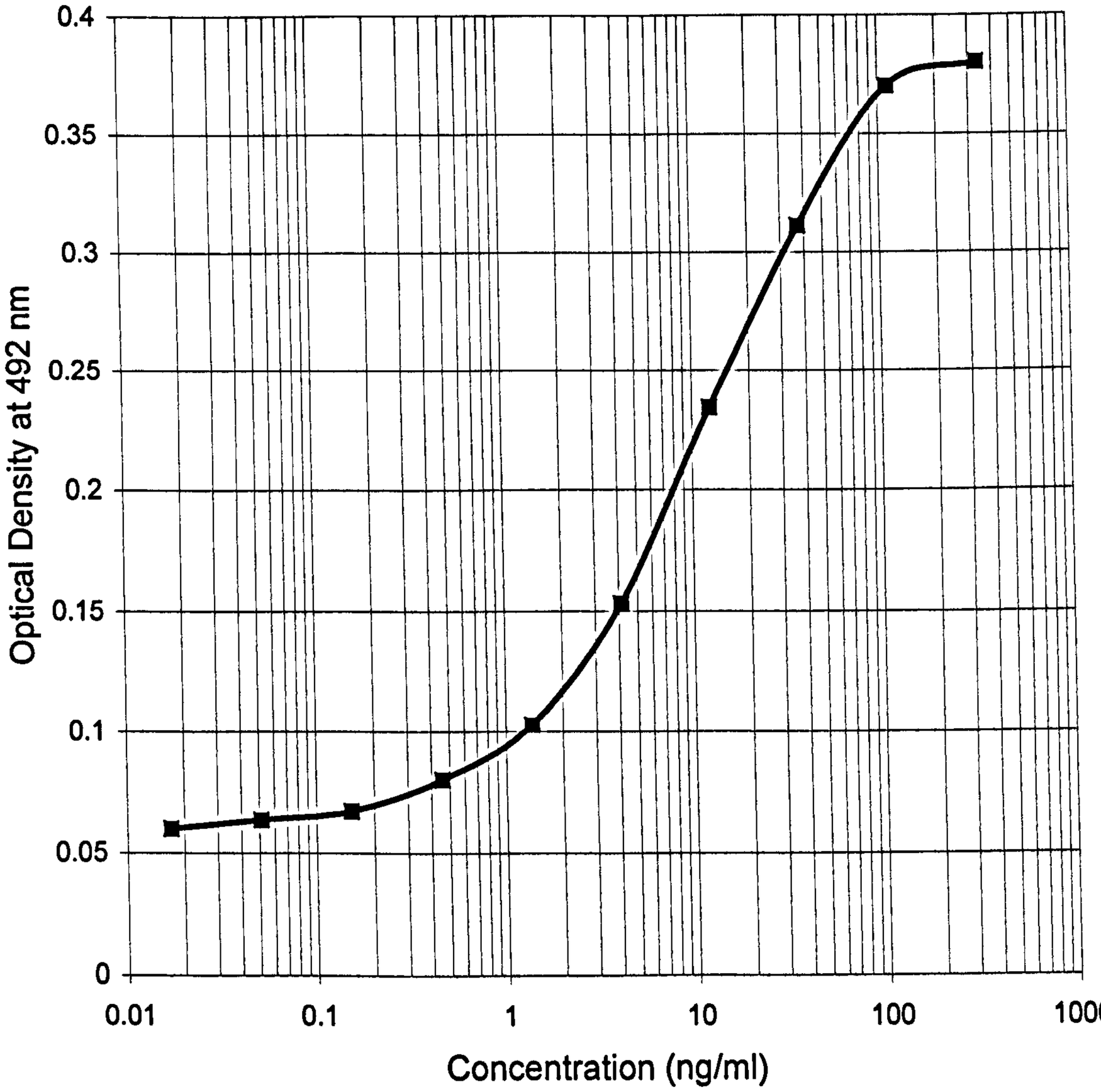


Figure 4.4 i
IgY-Fc ELISA standard curve

Typical ELISA standard curve used to determine IgY-Fc concentration in CHO-K1 culture supernatants. Known quantities of papain cleaved IgY-Fc (see chapter 3) were assayed in triplicate and mean optical densities at 492nm plotted as a function of log [IgY-Fc concentration in ng/ml]. A standard curve was generated for every ELISA plate and these data were obtained when the samples from the CHO transfections were tested (shown in table 4.4 ii).

Vector	Expression of IgY-Fc (ng/1x10^6 cells/ml) Transfection 1	Transfection 2
pATY24[wt]	309	217
pATY24[N308Q]	192	256
pATY24[N308Q N407Q]	100	120
pATY34[wt]	72	128
pATY34[C340S]	192	240
pATY34[C347S]	102	68
pATY34[C340S C347S]	125	133

Table 4.4 ii
Yields of secreted IgY-Fc mutants from transiently transfected CHO-K1 cells

Two separate transfections were performed using each vector and specific production rates of transiently expressing cells determined by ELISA.

4.5 Discussion

Vectors encoding a series of IgY-Fc mutants were designed, constructed and tested in order to produce recombinant fragments for structural and functional studies. Expression vectors for the complete IgY-Fc (Cυ2-4) were created and found to successfully induce secretion of fully glycosylated, partially glycosylated (N308Q) and aglycosylated (N308Q N407Q) forms from mammalian cells.

Glycosylation of IgG-Fc is an absolute requirement for receptor interaction. Receptor binding sites are disrupted due to changes in quaternary structure that occur when oligosaccharide is removed (Krapp *et al.*, 2003; Wright and Morrison, 1997). By contrast, deglycosylated IgE-Fc retains substantial receptor binding capability, suggesting that binding site architecture may be less affected by oligosaccharide removal (Bjorklund *et al.*, 1999). However, expression of aglycosylated IgE-Fc results in a fragment that lacks receptor binding capability (Nettleton and Kochan, 1995), which could indicate a role in initial folding of the IgE-Fc, but not maintenance of structure post-secretion. The structural similarities between IgY and IgE suggest that glycosylation of N407 in Cυ3, the homologous residue to N394 in Cε3, may play a similar role in IgY and IgE. However, an anti-IgY reactive product was detected in the supernatant of CHO cells transfected with the expression vector for aglycosylated Cυ2-4 [N308Q N407Q]. Whether this fragment is produced by stably transfected cells and is indeed aglycosylated will be discussed in subsequent chapters.

A series of vectors were created that encode IgY-Fc (Cυ3-4) with every combination of cysteine to serine mutations made at positions 340 and 347. All vectors were found to be capable of inducing expression of material from mammalian cells that was detectable by IgY-specific ELISA.

The full length and truncated IgY-Fc fragments will allow comparative studies of the IgY-Fc with and without the Cυ2 domains. Although very little is known how many types of IgY receptor exist in chicken and how they interact with the Fc, structural and functional analysis of the cysteine to serine mutants may allow the influence of putative interchain disulphide bonds to be studied.

The levels of expression achieved with transient transfections of CHO-K1 cells were impractical for further analysis of the recombinant IgY-Fcs. The CHO system, which can be highly productive for certain proteins (Cockett *et al.*, 1990), has previously been found to give poor yields of IgE-Fc Cε2-4 (Young, 1994). Stable secretors of IgY-Fc capable of producing milligram quantities of protein for techniques such as X-ray crystallography were required, so CHO cells were used solely as a means to verify the correct assembly and viability of the pATY expression vectors.

Chapter 5

Recombinant IgY-Fc Production, Purification & Initial Structural Characterisation

5.1 Overview

Although sub-fragments of immunoglobulins have been produced in bacteria (Boss *et al.*, 1984; Ishizaka *et al.*, 1986; Kenten *et al.*, 1984), yeast (Eldin *et al.*, 1997) and baculovirus-infected insect cells (Bjorklund *et al.*, 2000; Wurzburg *et al.*, 2000), mammalian expression systems are often favoured in structural studies (Basu *et al.*, 1993; Young *et al.*, 1995) as they avoid any potentially problematic refolding or post-translational modification issues that can occur in prokaryotic systems. In the previous chapter, a series of plasmid vectors were constructed to allow expression of recombinant IgY-Fc Cυ2-4, Cυ3-4 and several mutants in a mouse myeloma cell line, NS-0 (Bebbington *et al.*, 1992). As described in chapter 4, this cell line was used to produce substantial quantities of various IgE-Fc fragments in previous reported studies (see section 4.1 for references). NS-0 cells lack glutamine synthetase (GS) and cannot grow in glutamine-free medium unless transfected with a vector which encodes the enzyme. This allows a cheap, non-toxic approach to selection of stable transfectants. The following chapter describes the production of IgY-Fc secreting cells and the subsequent purification and initial characterisation of recombinant IgY-Fc fragments.

5.2 Stable expression of IgY-Fc fragments from NS-0 cells

5.2.1 Transfection and selection of stable NS-0 clones

NS-0 cells were grown in antibiotic-free medium to ~70-90% confluency before being transfected with a linearised pATY vector (see chapter 2). Stable transfectants were obtained by growth in glutamine-free selection medium and clones isolated by transfer of single colonies to new wells containing selection medium. Approximately 50-100 colonies were obtained per transfection, although only ~50% were in separate wells and were considered clonal. Typically ~25% of these clones would either fail to grow after transfer or would grow too slowly to be useful. Spent culture media of successfully propagated clones were harvested and assayed for the presence of IgY-Fc by ELISA (see chapter 2). Clones producing the highest levels of IgY-Fc were maintained in selection medium and subjected to further rounds of screening to ensure stability of expression. The majority of clones either lost expression (and/or died) after several weeks in culture or produced levels of IgY-Fc too low to be detected by ELISA. The remaining clones (typically fewer than 5) were then tested for specific production rate (see chapter 2) and aliquots stored in liquid nitrogen. The highest producers (Table 5.2.1) were expanded and used for subsequent IgY-Fc production. Transfections yielding no stable clones were repeated.

IgY-Fc	Mutation	Clone	Specific Production Rate ($\mu g/10^6$ cells/24hrs)
Cu2-4	wt	2-3 C12	8.5
	N308Q	1-2 B12	20.1
	N308Q N407Q	2-2 A10	1.5
Cu3-4	wt	2-3 B11	6.0
	C340S	3-3 H10	7.0
	C347S	3-1 H10	7.0
	C340S C347S	1-1 C11	7.8

Table 5.2.1
Specific IgY-Fc production rates of the best stable NS-0 transfectants

NS-0 cells were transfected with the pATY vectors (described in chapter 4), which encode the various IgY-Fc constructs. Successful transfectants were expanded, maintained and screened for secretion of IgY-Fc using anti-IgY ELISA of culture media. Data for the clones with the highest specific production rate of each IgY-Fc construct are shown. These clones were used to produce all IgY-Fc fragments used in subsequent experiments.

5.2.2 Growth of stable NS-0 clones and analysis of IgY-Fc secretion

The stable NS-0 clones were only partially adherent, so could be transferred to one litre roller bottles without lengthy adaptation to growth in suspension. Growth and expression patterns of NS-0 cells that secrete IgE-Fc have been previously determined (R.J. Young, 1994, PhD Thesis). Cells were found to reach confluency after six days post seeding, but IgE-Fc continued to accumulate with minimal degradation for about ten days. Quantity and quality of

secreted IgY-Fc was determined over the same time-scale using SDS-PAGE and Western blotting (Figure 5.2.2 i). The concentration of IgY-Fc appeared to plateau around 8 days post seeding, with no degradation apparent after 10 days, at which time no viable cells could be detected using trypan blue exclusion (data not shown). The concentration of each IgY-Fc accumulated was determined by ELISA (Table 5.2.2 ii). The rate of cell growth in rolling culture was not equal for all clones, so in some cases clones were able to accumulate more IgY-Fc than others whose specific production rates were apparently higher. Despite this, all IgY-Fc mutants were produced in sufficient quantities for further studies.

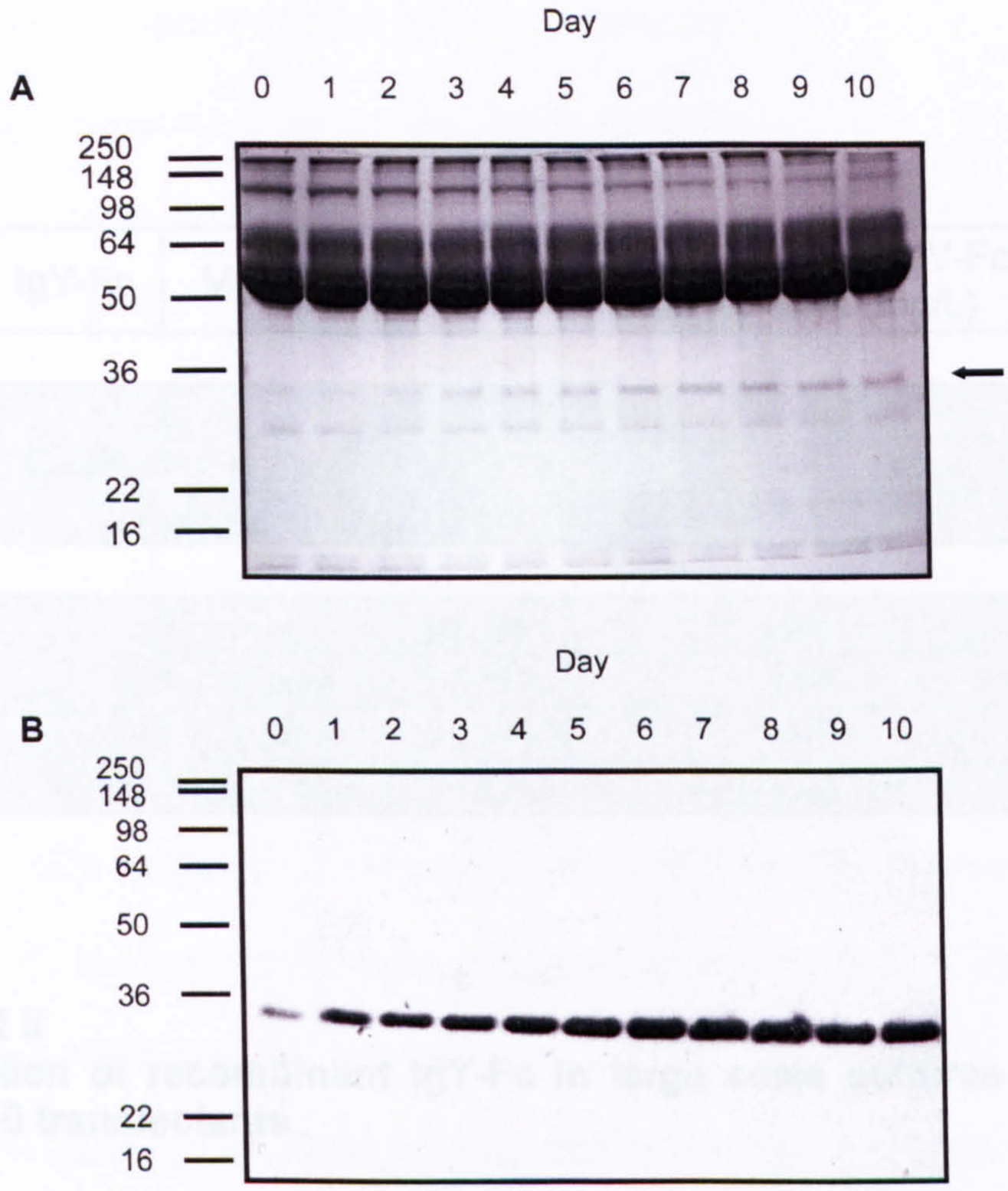


Figure 5.2.2 i
Accumulation of recombinant IgY-Fc in stable NS-0 transfectant 1-1 C11

Samples of medium were taken every 24 hours from stable NS-0 transfectants growing in roller bottles for 10 days and analysed by SDS-PAGE using a non-native 12% polyacrylamide gel. The samples were either stained with (A) Coomassie or transferred to nitrocellulose for (B) Western blotting using a goat polyclonal anti-IgY antibody. Although the culture medium contained numerous proteins, only one detectable ~30kDa band appeared over the course of the culture (marked). Only bands at this molecular weight were detected by anti-IgY Western blot. ~30Kda is consistent with a Cυ3-4 fragment that is monomeric upon denaturation, which this clone (1-1 C11) was designed to produce. Like all of the recombinant IgY-Fc fragments produced (not shown), Cυ3-4 [C340S C347S] showed maximal production at or around day 8 and minimal degradation even after 10 days.

IgY-Fc	Mutation	Clone	Concentration of IgY-Fc after 10 days (mg/L)
Cυ2-4	wt	2-3 C12	31
	N308Q	1-2 B12	340
	N308Q N407Q	2-2 A10	16
Cυ3-4	wt	2-3 B11	94
	C340S	3-3 H10	260
	C347S	3-1 H10	48
	C340S C347S	1-1 C11	250

Table 5.2.2 ii
Accumulation of recombinant IgY-Fc in large scale cultures of the best stable NS-0 transfectants

The stable NS-0 transfectants with the highest specific IgY-Fc production rate were chosen to seed larger scale cultures in non-selective medium. The cultures were incubated for 10 days before supernatants were harvested and the concentration of accumulated IgY-Fc measured by ELISA.

5.3 Purification of recombinant IgY-Fc fragments

5.3.1 Affinity purification of recombinant IgY-Fc fragments

Supernatants from NS-0 cultures were harvested by centrifugation and sterile filtered. IgY-Fc was purified using an affinity column packed with rabbit anti-IgY antibodies coupled to agarose (see chapter 2). Although the capacity of the beads for whole IgY was 1.2mg/ml, the capacity for IgY-Fc was found to be ~0.2mg/ml. For practical reasons, only ~20mg was purified from each supernatant. The majority of impurities were removed by a single bind/wash/elute cycle, although traces of serum proteins were detected on an overloaded SDS-PAGE gel. Impurities were estimated by densitometry to be less than 1%, but for experiments where purity and accurate determination of concentration were crucial, homogeneity was further improved by a second affinity purification cycle. Although this method would not remove aggregated IgY-Fc, the quantity of large aggregates was determined to be negligible by UV absorbance at >320nm.

5.3.2 Further purification of recombinant IgY-Fc fragments by size exclusion

Due to time constraints, only one of the IgY-Fc mutants prepared in 5.3.1 was selected for further purification by size exclusion chromatography in order to begin initial screening for crystallisation conditions. Cυ2-4 [N308Q] was chosen as this mutant is homologous to the recombinant IgE-Fc used to determine the Cε2-4 crystal structure (Wan *et al.*, 2002). HPLC separated fractions containing the IgY-Fc, as determined by western blot (not shown), were pooled and re-analysed to confirm homogeneity (Figure 5.3.2).

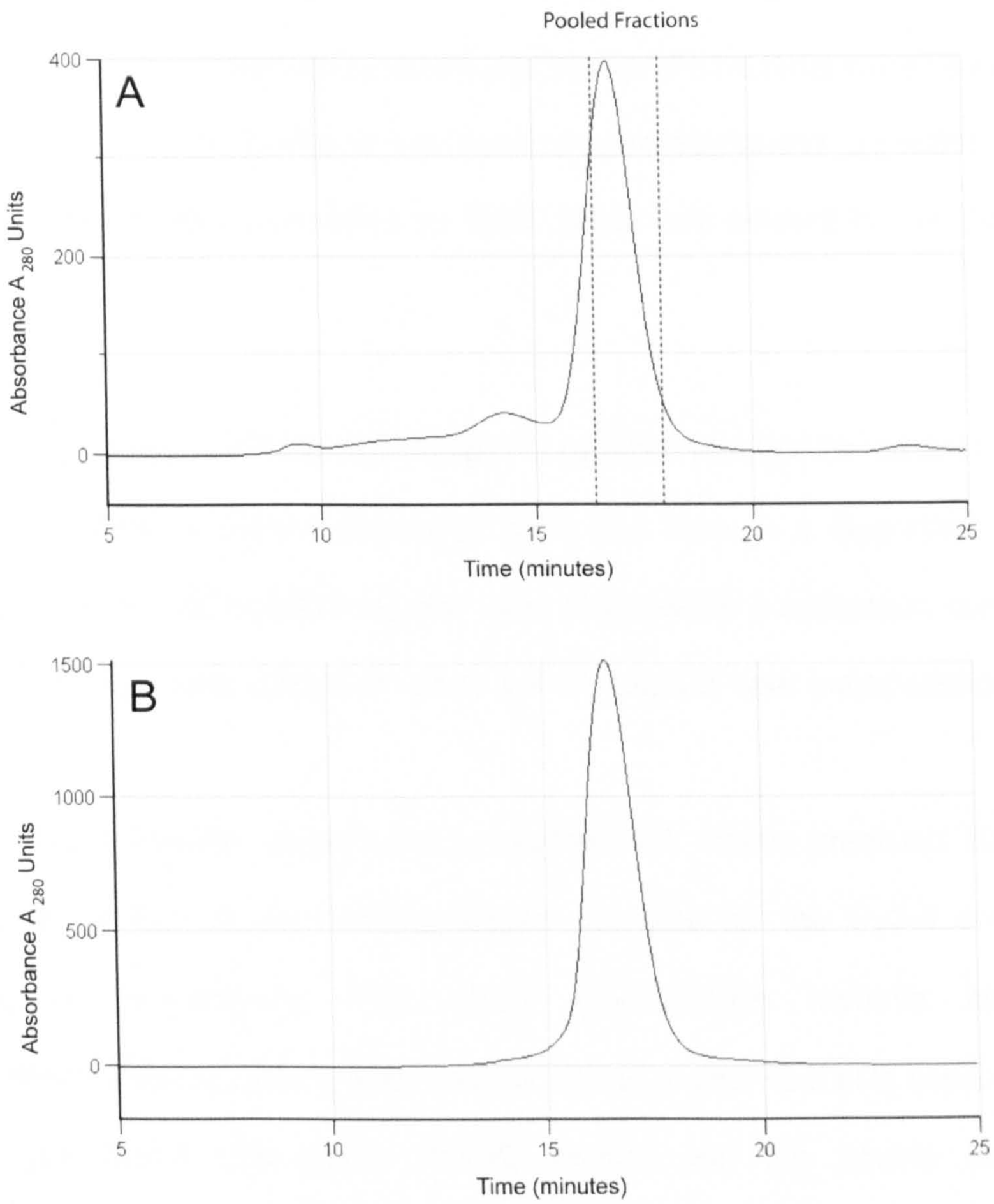


Figure 5.3.2
Purification of IgY-Fc Cυ2-4 [N308Q] by gel filtration chromatography

Gel filtration profiles of double affinity purified IgY-Fc Cυ2-4 [N308Q], before (A) and after (B) further purification by size exclusion using a Superdex 200 column (GE Healthcare).

5.4 Initial structural characterisation of recombinant IgY-Fc mutants

Some basic structural properties of the IgY-Fc mutants were investigated in order to assess the quality of the recombinant material and to confirm that the intended modifications encoded by each vector are evident in the expressed proteins.

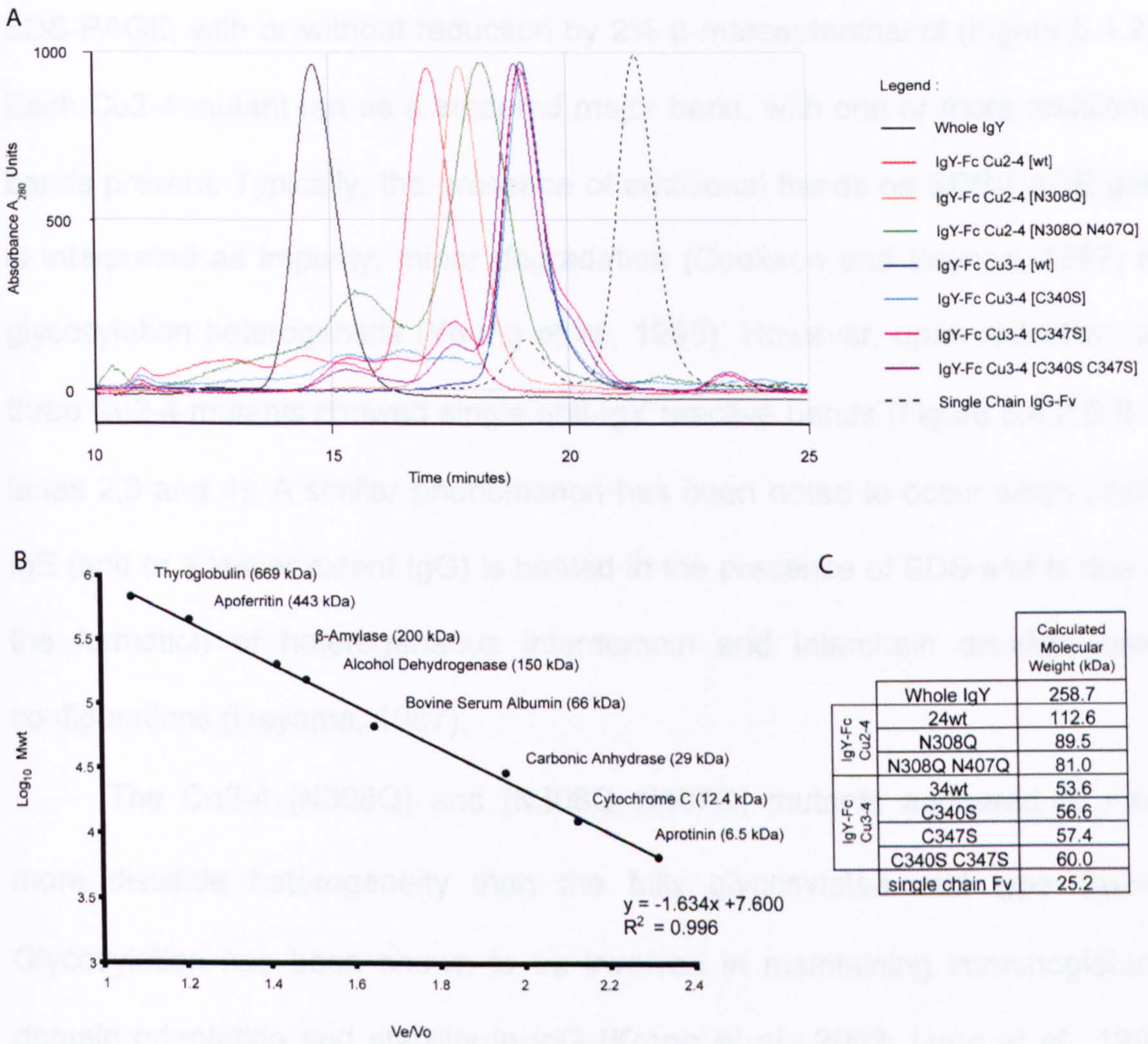
5.4.1 Determination of molecular weight of recombinant IgY-Fc mutants

A series of protein standards were run through a Superdex 200 gel filtration matrix (GE Healthcare) and used to generate a calibration curve, from which the molecular weight of each IgY-Fc mutant was extrapolated (Figure 5.4.1).

The molecular weights are consistent with values predicted for dimers made up of four or six immunoglobulin domains for the Cυ3-4 and Cυ2-4 fragments, respectively. The Cυ2-4 glycosylation mutants could be discriminated easily, with a difference of ~23 kDa and ~8.5 kDa separating the wild type Cυ2-4, the single N308Q mutant and the double (ostensibly aglycosylated) mutant. This implies that the oligosaccharide attached to asparagine 308 accounts for ~11.5 kDa per chain, whereas the oligosaccharide on asparagine 407 accounts for only ~4.25 kDa per chain. Glycosylation of IgY-Fc is reportedly complex type at N308 and high-mannose at N407 (Suzuki and Lee, 2004). Complex oligosaccharides have longer terminal regions than high-mannose, but high mannose has more branching so overall their sizes are similar (Hubbard and Ivatt, 1981). The difference in apparent molecular weight is likely due to the location of the glycosylation sites, which may differentially affect the overall shape of the Fc, and therefore gel retention time; if the sites in

IgY-Fc are equivalent to their IgE counterparts, the carbohydrate attached to N308 will be on the outer face of Cυ2 so may provide a greater contribution to shape than N407-linked oligosaccharide, which may protrude into the core of the Fc.

The Cυ3-4 mutants were found to have similar molecular weights, including the mutant lacking interchain disulfide bonds, Cυ3-4 [C340S / C347S]. This suggests that IgY-Fc is able to dimerise even in the absence of interchain disulphide bridges, as is the case for Fc regions of both IgG (Aschaffenburg *et al.*, 1979; Fleischman *et al.*, 1963) and IgE (Basu *et al.*, 1993; Hunt *et al.*, 2005). The Cγ3:Cγ3 and Cε4:Cε4 domain pairs form extensive (primarily hydrophobic) interactions (Deisenhofer, 1981; Wan *et al.*, 2002), which are thought to provide the platform for dimerisation of the complete Fc. Conservation of these non-covalent interactions from IgY to IgG and IgE would imply crucial structural or functional importance, although these interactions may be weaker in IgY (Dreesman and Benedict, 1965b)



		Calculated Molecular Weight (kDa)
	Whole IgY	258.7
IgY-Fc Cu2-4	24wt	112.6
	N308Q	89.5
	N308Q N407Q	81.0
IgY-Fc Cu3-4	34wt	53.6
	C340S	56.6
	C347S	57.4
	C340S C347S	60.0
	single chain Fv	25.2

Figure 5.4.1
Estimation of the molecular weight of the IgY-Fc mutants using size exclusion chromatography

(A) Gel filtration profile of IgY and IgY-Fc mutants. The volume necessary to elute each protein (V_e), divided by the dead volume of the column (V_0) was compared to a calibration curve of standards eluted from the same column in the same buffer (B), in order to extrapolate the molecular weight for IgY and each IgY-Fc mutant (C). An antibody fragment comprised of only two immunoglobulin domains, single chain IgG-Fv (prepared by Dr. H. Harries and Dr. A. Davies), was included to show that the IgY-Fc fragment molecular weights are consistent with dimers of four (Cυ3-4) or six (Cυ2-4) immunoglobulin domains. NB. A different S200 column was used in this experiment than those reported in previous figures.

5.4.2 Determination of relative mobility of recombinant IgY-Fc mutants

The relative mobility of each IgY-Fc mutant was assessed by denaturing SDS-PAGE, with or without reduction by 2% β-mercaptoethanol (Figure 5.4.2). Each Cυ2-4 mutant ran as a smeared major band, with one or more additional bands present. Typically, the presence of additional bands on SDS-PAGE gels is interpreted as impurity, minor degradation (Cookson and Beynon, 1987) or glycosylation heterogeneity (Young *et al.*, 1995). However, upon reduction, all three Cυ2-4 mutants showed single anti-IgY reactive bands (Figure 5.4.2 B & D lanes 2,3 and 4). A similar phenomenon has been noted to occur when whole IgE (and to a lesser extent IgG) is heated in the presence of SDS and is due to the formation of heterogeneous interdomain and interchain disulfide bond configurations (Ikeyama, 1987).

The Cυ2-4 [N308Q] and [N308Q N407Q] mutants appeared to have more disulfide heterogeneity than the fully glycosylated wild type Cυ2-4. Glycosylation has been shown to be involved in maintaining immunoglobulin domain orientation and stability in IgG (Krapp *et al.*, 2003; Lund *et al.*, 1990; Mimura *et al.*, 2001) and, albeit less crucially, IgE (Bjorklund *et al.*, 1999). It is conceivable that even minor structural changes brought about by the removal of N-linked glycosylation could affect which sets of interdomain or interchain disulfides are possible or more energetically favourable. Whether these changes occur in the native protein or are simply an artefact of SDS-PAGE (Kumar *et al.*, 1993), remains to be seen.

SDS-PAGE analysis of the Cυ3-4 mutants confirmed the lack of interchain disulfides in the [C340S / C347S] mutant; the denatured fragment had a mobility consistent with a monomer and was unaffected by reduction

(Figure 5.4.2 lane 8). Differences in migration between the unreduced [C340S] and [C347S] mutants, but not their reduced forms (Figure 5.4.2 lanes 6 & 7), further highlight the influence of disulfide bonds on electrophoretic mobility.

The analysis also showed that approximately half of the wild type Cυ3-4, which was expected to possess two interchain disulfide bonds and therefore dimerise, in fact has a mobility consistent with a monomer (Figure 5.4.2 lane 5). It is likely that the S-S bonds have either failed to form, or have formed in an intrachain fashion, resulting in a lack of interchain covalent bonds. There is some evidence for the necessity of contacts from other domains in the IgE-Fc heavy chain for folding of Cε3 to occur efficiently (J. Hunt, 2004, PhD Thesis). In IgY-Fc, the presence of Cυ2 may be required to prevent misfolding and/or incorrect pairing of cysteines at the Cυ2/Cυ3 interface. It is worth noting that even if a disulfide bond forms between cysteines at position 340, as may initially be expected, this could be artefactual. The 'correct' pairing in the full IgY-Fc may in fact be a crossed disulfide between C340 and C252 on the opposite heavy chain, as is the case for the homologous residues in Cε2, C328 and C241 respectively (Wan *et al.*, 2002). Efforts were made to separate the two species, but they could not be differentiated on the basis of size, charge or affinity for immobilised anti-IgY antibodies. It was decided that the protein would be used in further investigations, but with the caveat that some artefacts may arise.

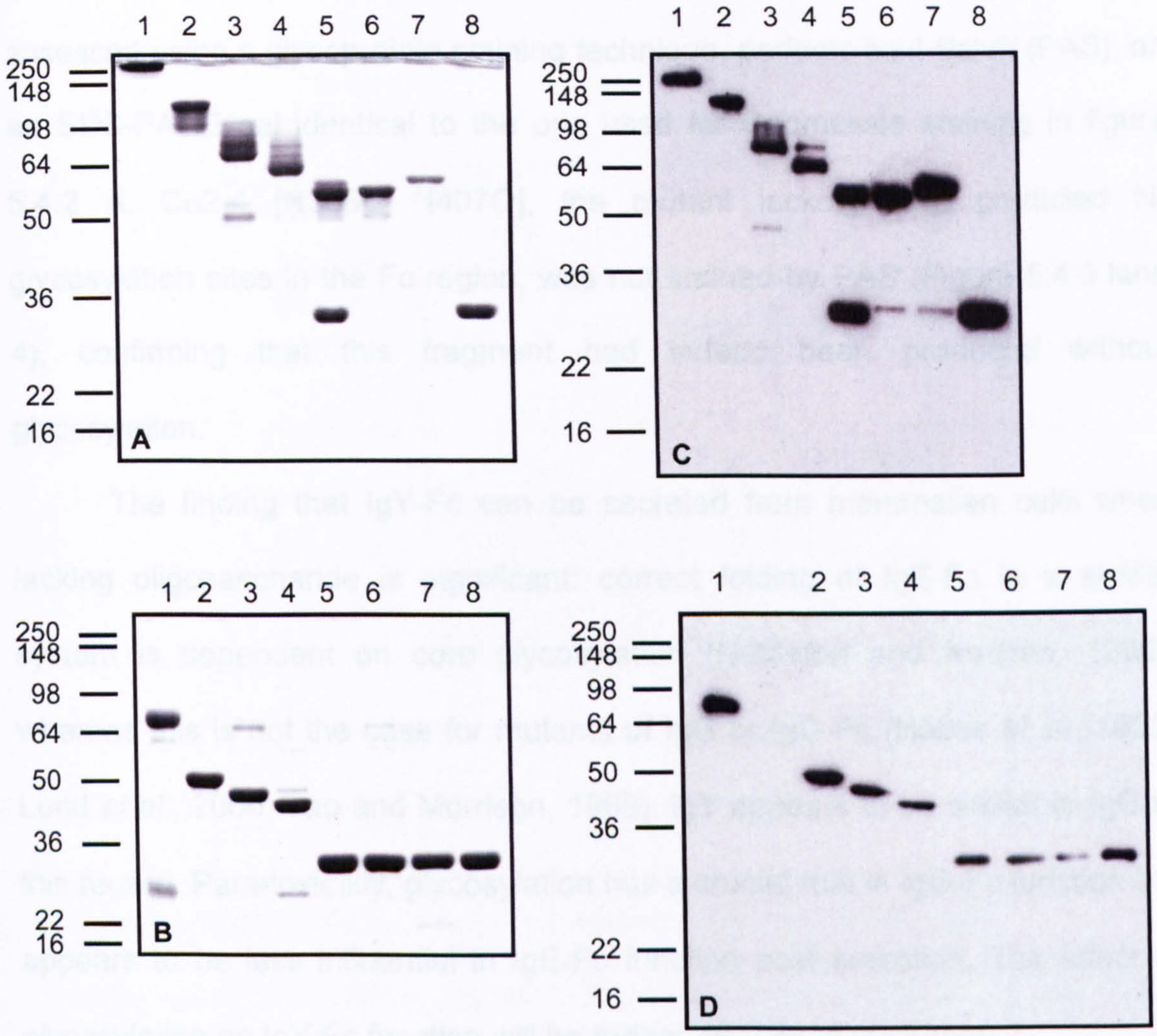


Figure 5.4.2
SDS-polyacrylamide gel electrophoresis and Western blot analysis of IgY-Fc fragments

Whole IgY and recombinant IgY-Fc fragments secreted from NS-0 cells were run on denaturing 12% polyacrylamide gels and either stained with Coomassie (A & B) or transferred to nitrocellulose and Western blotted with polyclonal anti-IgY antibodies (C & D). Samples were either non-reduced (A & C) or reduced with 2% β–mercaptoethanol (B & D). All gels were loaded as follows: **Lane 1**, IgY, whole molecule. **Lane 2**, Cυ2-4 wild type. **Lane 3**, Cυ2-4 [N308Q]. **Lane 4**, Cυ2-4 [N308Q N407Q]. **Lane 5**, Cυ3-4 wild type. **Lane 6**, Cυ3-4 [C340S]. **Lane 7**, Cυ3-4 [C347S]. **Lane 8**, Cυ3-4 [C340S C347S]. Molecular weight markers are shown in kDa.

5.4.3 Determination of recombinant IgY-Fc glycosylation state

The carbohydrate content of the IgY-Fc mutants was qualitatively assessed using a glycoprotein staining technique, periodic acid-Schiff (PAS), on an SDS-PAGE gel identical to the one used for Coomassie staining in figure 5.4.2 A. Cυ2-4 [N308Q N407Q], the mutant lacking both predicted N-glycosylation sites in the Fc region, was not stained by PAS (Figure 5.4.3 lane 4), confirming that this fragment had indeed been produced without glycosylation.

The finding that IgY-Fc can be secreted from mammalian cells when lacking oligosaccharide is significant: correct folding of IgE-Fc in a similar system is dependent on core glycosylation (Nettleton and Kochan, 1995), whereas this is not the case for mutants of IgG or IgG-Fc (Hobbs *et al.*, 1992; Lund *et al.*, 2000; Tao and Morrison, 1989). IgY appears to be similar to IgG in this regard. Paradoxically, glycosylation has a crucial role in IgG-Fc function but appears to be less influential in IgE-Fc function post secretion. The effect of glycosylation on IgY-Fc function will be further discussed in chapter 7.

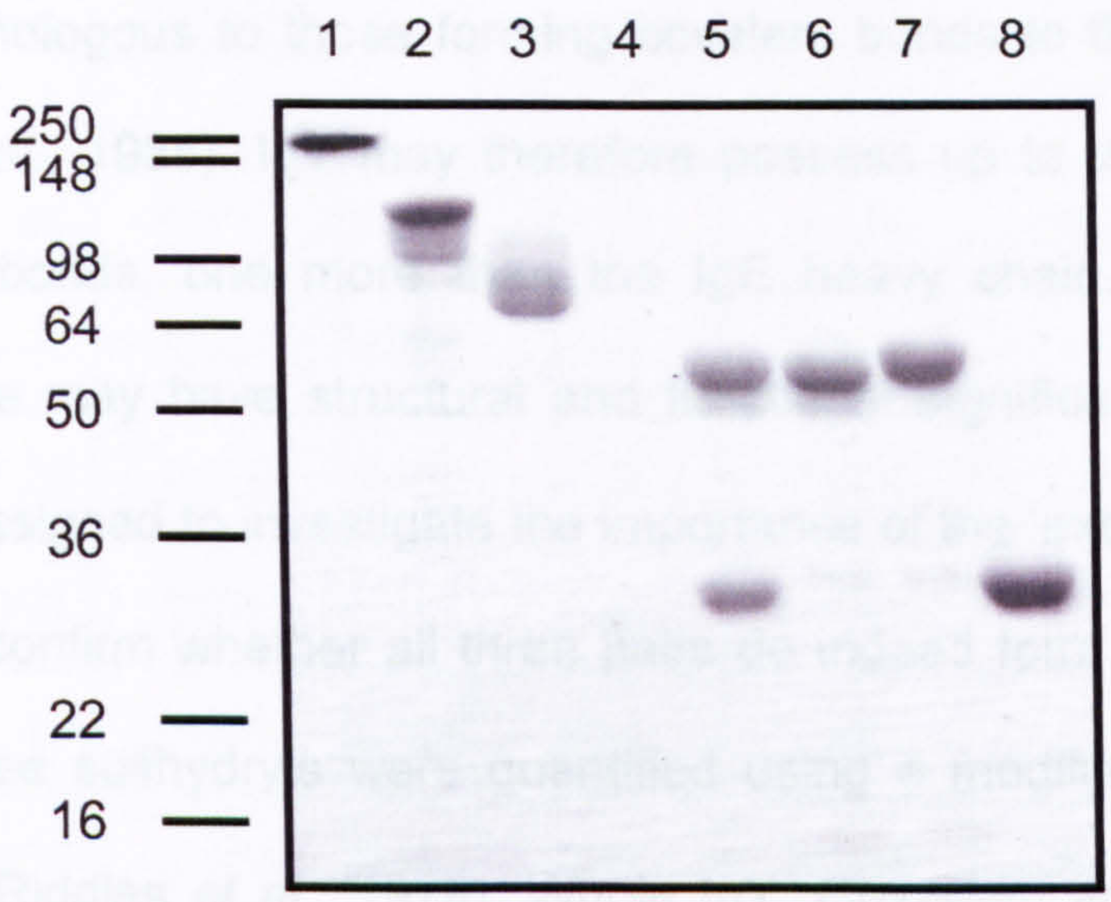


Figure 5.4.3
Qualitative assessment of IgY-Fc fragment glycosylation state

Periodic acid-Schiff (PAS) stained 12% non-reduced SDS-PAGE gel. 10µg protein loaded per well. **Lane 1**, IgY, whole molecule. **Lane 2**, Cυ2-4 wild type. **Lane 3**, Cυ2-4 [N308Q]. **Lane 4**, Cυ2-4 [N308Q N407Q]. **Lane 5**, Cυ3-4 wild type. **Lane 6**, Cυ3-4 [C340S]. **Lane 7**, Cυ3-4 [C347S]. **Lane 8**, Cυ3-4 [C340S C347S]. Molecular weight markers are shown in kDa.

5.4.4 Quantification of free sulfhydryls in IgY and recombinant IgY-Fc

The upsilon heavy chain of IgY has been found to contain three pairs of cysteine residues not predicted to be involved in intradomain bonds, in addition to cysteines orthologous to those forming covalent bonds to the light chain in IgE (Parvari *et al.*, 1988). IgY may therefore possess up to three inter-heavy chain disulfide bonds, one more than the IgE heavy chain. The additional putative disulfide may have structural and functional significance. The Cυ3-4 mutants were designed to investigate the importance of the 'extra' cysteine pair, but in order to confirm whether all three pairs do indeed form disulfides in the complete Fc, free sulfhydryls were quantified using a modified Ellman assay (Ellman, 1958; Riddles *et al.*, 1979). Whole IgY, Cυ2-4[wt] and Cυ3-4[C340S C347S] were assayed (Table 5.4.3). Bovine Serum Albumin (BSA), which contains one free sulfhydryl, was included as a positive control and human IgG and IgG-Fc, which contain none, as negative controls.

The free sulfhydryl:protein ratios of whole IgY and both fragments assayed were well below the control value, which indicated that they contained no free sulfhydryls. The Cυ3-4[C340S C347S] mutant lacks any predicted interchain disulfides, and was included as an additional negative control. The results for whole IgY and Cυ2-4[wt] appear to confirm that all three interchain disulfides do indeed form, assuming that they would otherwise be free sulfhydryls.

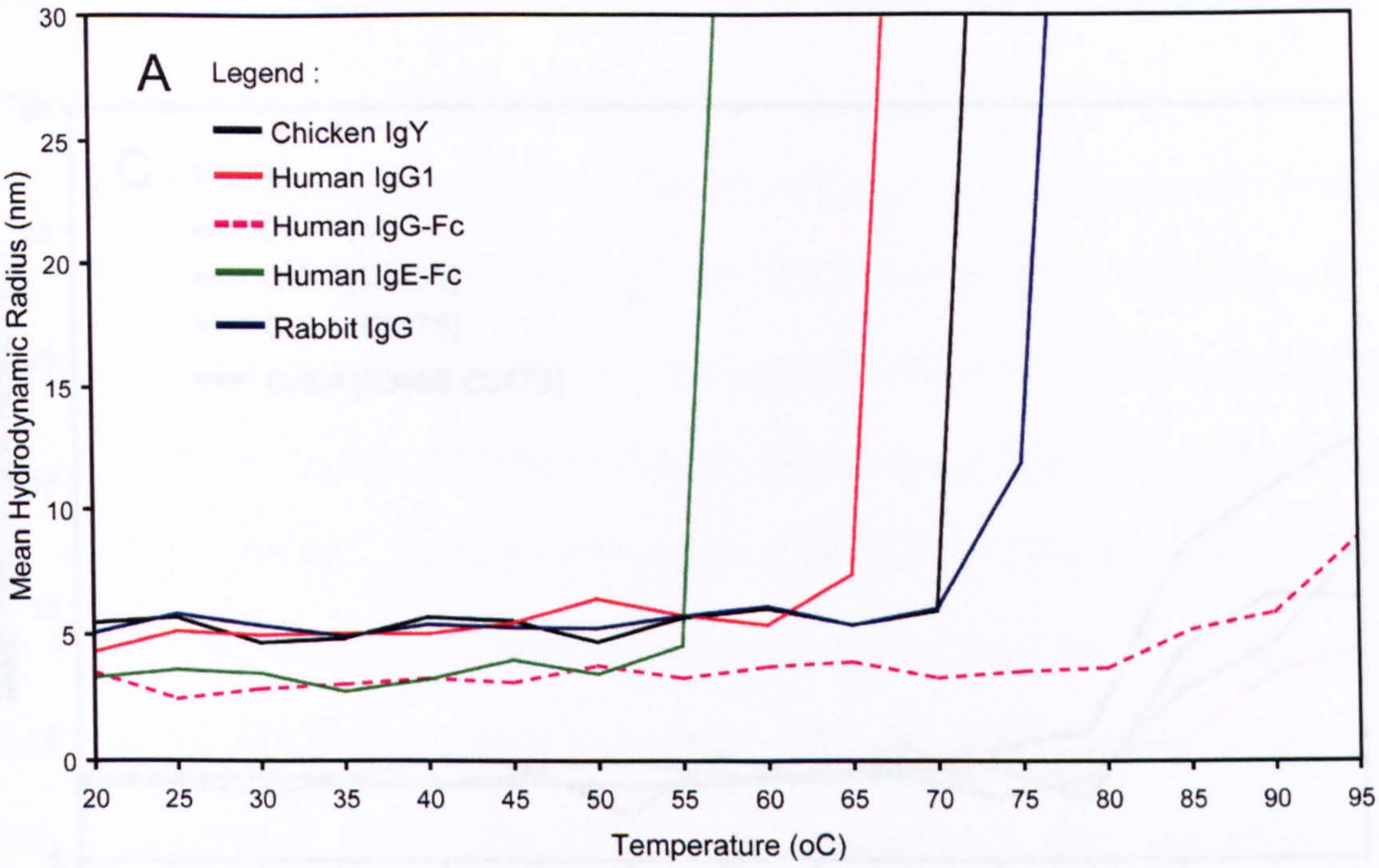
	A_{280}	TNB A_{280} contribution	A_{412}	TNB molarity (µmol L^{-1})	Protein molar extinction coefficient	Protein molarity (µmol L^{-1})	Free sulfhydryl : protein ratio
BSA (control)	0.94	0.016	0.204	14.42	43824	20.96	0.69
IgG1	0.49	0.009	0.001	0.07	223400	2.16	0.03
IgG1-Fc	0.79	0.014	0.001	0.07	71570	10.82	0.01
IgY	0.55	0.010	0.001	0.06	209125	2.58	0.02
Cυ2-4 [wt]	0.35	0.006	0.005	0.34	90965	3.78	0.09
Cυ3-4 [C340S C347S]	0.50	0.009	0.001	0.07	62380	7.91	0.01

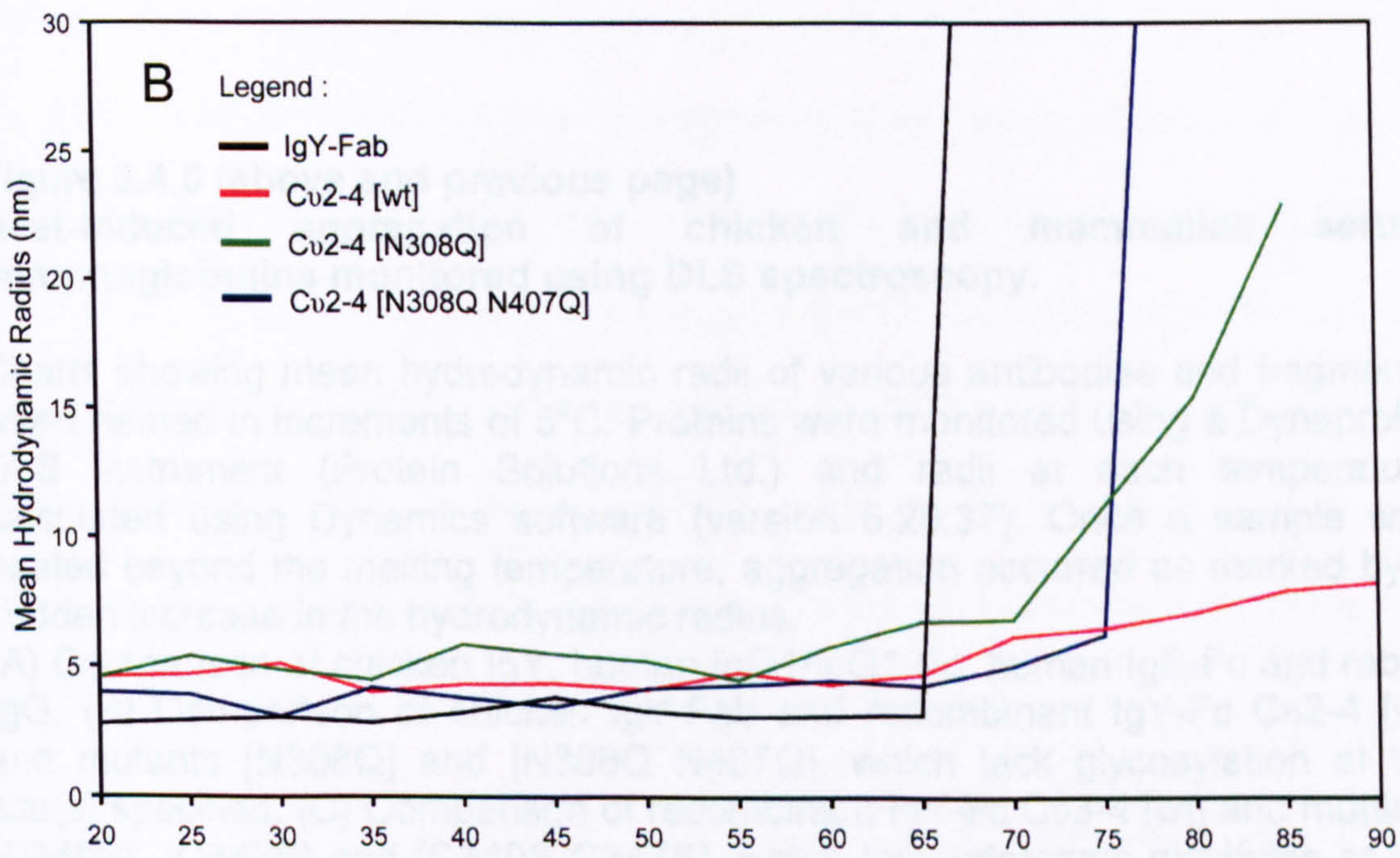
Table 5.4.4
Comparison of free sulfhydryls in chicken and human serum immunoglobulins and their Fc fragments

The number of free sulfhydryls was calculated by reacting them with Ellman's reagent, DTNB, then using photospectrometry to measure a coloured product released upon strong reduction. The value obtained for the positive control, BSA, is typical for this assay (Riener *et al.*, 2002) and should be interpreted as one free sulfhydryl per molecule of BSA.

5.4.5 Heat-induced aggregation of IgY and recombinant IgY-Fc

Dynamic light scattering (DLS) was used to evaluate thermal stability of IgY, IgG, IgE and/or their Fc fragments (see chapter 2). As an immunoglobulin unfolds, core hydrophobic regions are exposed leading to irreversible aggregation (Vermeer and Norde, 2000), which can be measured by an increase in the mean hydrodynamic radius of particles in solution. DLS does not directly measure unfolding, as more sophisticated methods like differential scanning calorimetry (DSC) or circular dichroism (CD) spectroscopy do, so detailed information about unfolding transitions of individual domains cannot be obtained. The technique does, however, provide an approximation of the 'melting' temperature (T_m) at which unfolding has proceeded to such an extent that aggregation occurs. The results are shown in figure 5.4.5.


A
Legend :
Chicken IgY
Human IgG1
Human IgG-Fc
Human IgE-Fc
Rabbit IgG
Mean Hydrodynamic Radius (nm)
Temperature (oC)


B
Legend :
IgY-Fab
Cυ2-4 [wt]
Cυ2-4 [N308Q]
Cυ2-4 [N308Q N407Q]
Mean Hydrodynamic Radius (nm)
Temperature (°C)

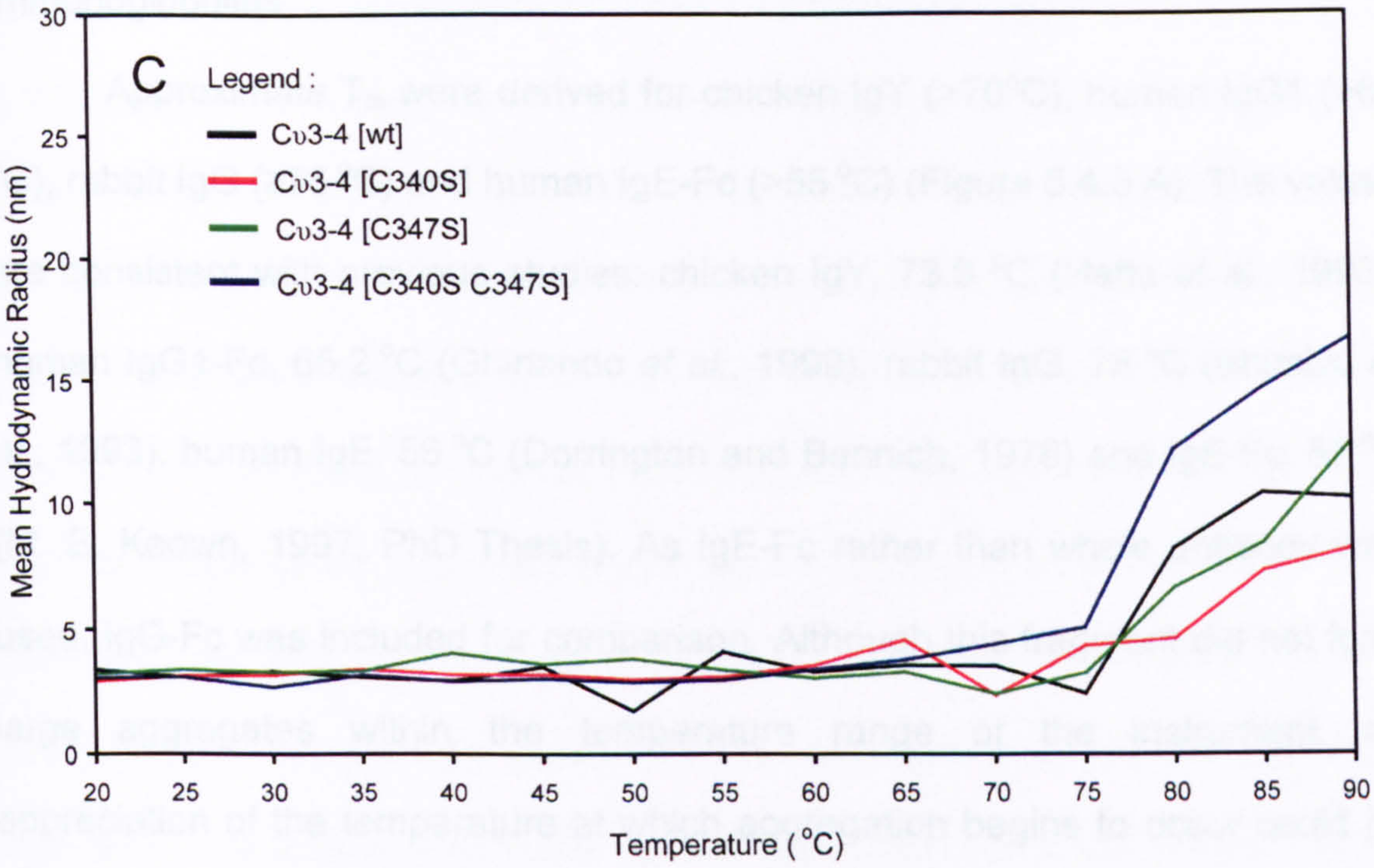


Figure 5.4.5 (above and previous page)
Heat-induced aggregation of chicken and mammalian serum immunoglobulins monitored using DLS spectroscopy.

Charts showing mean hydrodynamic radii of various antibodies and fragments when heated in increments of 5°C. Proteins were monitored using a Dynapro99 DLS instrument (Protein Solutions Ltd.) and radii at each temperature calculated using Dynamics software (version 5.26.37). Once a sample was heated beyond the melting temperature, aggregation occurred as marked by a sudden increase in the hydrodynamic radius.
(A) Comparison of chicken IgY, human IgG1/IgG1-Fc, human IgE-Fc and rabbit IgG. (B) Comparison of chicken IgY-Fab and recombinant IgY-Fc Cυ2-4 [wt] and mutants [N308Q] and [N308Q N407Q], which lack glycosylation at the site(s) specified. (C) Comparison of recombinant IgY-Fc Cυ3-4 [wt] and mutants [C340S], [C347S] and [C340S C347S], which lack interchain disulfides at the residue(s) specified.

5.4.5.1 Relative melting temperatures (T_m) of chicken and mammalian serum immunoglobulins

Approximate T_m were derived for chicken IgY (>70°C), human IgG1 (>65 °C), rabbit IgG (≥75 °C) and human IgE-Fc (>55 °C) (Figure 5.4.5 A). The values are consistent with previous studies: chicken IgY, 73.9 °C (Hatta *et al.*, 1993), human IgG1-Fc, 65.2 °C (Ghirlando *et al.*, 1999), rabbit IgG, 78 °C (Shimizu *et al.*, 1993), human IgE, 56 °C (Dorrington and Bennich, 1978) and IgE-Fc, 57 °C (M. B. Keown, 1997, PhD Thesis). As IgE-Fc rather than whole antibody was used, IgG-Fc was included for comparison. Although this fragment did not form large aggregates within the temperature range of the instrument, an appreciation of the temperature at which aggregation begins to occur could be observed: ~80 °C, which fits with the temperature of the second transition (Cγ3 melting) seen in microcalorimetry experiments, 83.3 °C at pH 8 (Tischenko *et al.*, 1998). Fab and recombinant Fc fragments of IgY were also tested (Figure 5.4.5 B). Like the homologous fragments of IgG (Vermeer and Norde, 2000), IgY-Fab (T_m >65 °C) was found to be less thermally stable than IgY-Fc (T_m >75 °C). Aggregation of whole IgY and IgG will therefore occur through the Fab region rather than the Fc, as this region would be the first to unfold as the temperature is increased. This represents a significant difference between IgY and IgE, which has potentially important consequences.

IgY and IgY-Fc appear to be more thermally stable than IgE-Fc; aggregation of IgY was found to occur at a higher temperature, in the same range as the mammalian IgGs tested. More than 80 years ago, IgE (then known as 'atopic reagin') was found to be susceptible to heat denaturation (Coca and Grove, 1925), more so than IgG. This has subsequently been shown to be due

to an unstable region in the Fc (Dorrington and Bennich, 1973). More recent studies using *E.coli* expressed individual Cε3 domains suggest that it exists as a 'molten globule', a state usually ascribed to protein folding intermediaries, with near-native secondary structure but poorly-defined tertiary structure (Price *et al.*, 2005; Ptitsyn, 1995). Data obtained using NMR has led McDonnell *et al.* to suggest that complete folding of Cε3 may only occur upon receptor binding (FcεRI, but not CD23), a process which may allow the specificity and affinity of the interaction to be optimised (Harwood *et al.*, 2006). In the context of the complete IgE, this flexibility could go some way towards explaining how a conformational change may occur, which is postulated to be responsible for the high affinity and slow dissociation of the IgE:FcεRI interaction (Harwood and McDonnell, 2007; Wan *et al.*, 2002). The IgY results show that the same degree of thermal instability is not present in the homologous Cυ3 domain, which suggests that this property was not found in the last common (IgY-like) ancestor of IgE and IgG. Receptor binding domain instability could therefore be one of the most important isotype-limited changes to have evolved since the divergence of the epsilon and gamma heavy chain genes.

5.4.5.2 The effect of Cυ2, glycosylation and interchain disulfide bonds on IgY-Fc melting temperature (T_m)

The contribution of the presence of various structural elements on thermal stability of the IgY-Fc was determined by comparison of the IgY-Fc mutants prepared in section 5.3 (Figure 5.4.5 B & C). As with IgG-Fc mentioned above, several of the mutants did not show as marked an increase in mean hydrodynamic radius as other proteins. The most likely explanation is that

conditions were not quite favourable for formation of larger aggregates within the temperature range of the instrument. The melting temperature is actually the point at which unfolding begins to occur, therefore any increase in the radius reflects a collapse of the least stable domain, which is useful for comparative purposes.

The position of interchain disulfide bonds (between cysteines at position 340 or 347) and the presence or absence of the Cυ2 domains appeared to have only minor effects on thermal stability, as Cυ2-4 [wt] and the relevant Cυ3-4 disulfide mutants appeared to have similar T_ms, all approximately 75°C (Figure 5.4.5 B & C). However, the T_m of the [C340S C347S] mutant appears to be slightly lower, indicating that the complete absence of interchain disulfide bonds had a deleterious effect on the thermal stability. The removal of interchain disulfide bonds produces very similar results with IgG-Fc and IgE-Fc: DSC experiments comparing deglycosylated IgG-Fc to deglycosylated, reduced and alkylated IgG-Fc revealed a drop in the T_m of Cγ2 from 65.7 °C to 63.9 °C (Mimura *et al.*, 2001). Likewise, the T_m of deglycosylated IgE-Fc (Cε3-4) and aglycosylated Cε3-4ΔC (lacking an interchain disulfide) showed a greater difference by CD spectroscopy, 53 °C and 48 °C respectively (J. Hunt, 2004, PhD Thesis).

The Cυ2-4 glycosylation site mutants showed striking differences in their behaviour upon heating. Although it was difficult to make an accurate determination of the T_m of the fully glycosylated [wt] mutant, comparison of the traces in figure 5.4.5 B show that the [N308Q N407Q] mutant (which is aglycosylated as confirmed in section 5.4.3) had a far greater propensity to

aggregate, as demonstrated by an abrupt increase in the mean hydrodynamic radius, at temperatures that appeared to have very little effect on the [wt] Cυ2-4.

Carbohydrate plays an important role in the maintenance of IgG-Fc structure as its removal causes a conformational compaction and a significant reduction in thermal stability (Ghirlando *et al.*, 1999; Krapp *et al.*, 2003; Mimura *et al.*, 2000). Similarly, removal of carbohydrate from IgE-Fc (Cε3-4) also reduces the thermal stability, although this is probably not accompanied by identical structural effects as Cε3-4 is already rather more compact compared to IgG-Fc (Wurzburg *et al.*, 2000). Although there are significant differences in the effect of carbohydrate on Fc folding (see section 5.4.3) and receptor binding (see chapter 7), the influence on stability and solubility would appear to be well conserved between IgG, IgE and IgY.

5.4.6 Determination of sensitivity of IgY interchain disulphide bonds to reduction by DTT

Interchain disulfides in the Fc region are not an absolute requirement for engagement of Fc receptor by IgE or IgG, although the affinity of the interaction is substantially weakened by their absence (Hunt *et al.*, 2005; McCool *et al.*, 1985). Rather than direct involvement in the Fc binding site, this is likely explained by changes in quaternary conformation. The Cγ2 and Cε3 domain pairs have little or no interdomain protein contacts (Deisenhofer, 1981; Wurzburg *et al.*, 2000), so their orientation relative to one another could be dramatically altered by removal of covalent bonds. The functional implications of this are discussed further in chapter 7.

Whilst inter-heavy chain disulfides may have similar functional roles in human IgE and IgG1, a significant difference is their susceptibility to reduction by DTT: 10mM is the minimum concentration required to reduce inter-heavy chain disulfides in IgE (Takatsu *et al.*, 1975), whereas less than half that is sufficient to have the same effect in IgG (Gall *et al.*, 1968). The disparity is likely due to the accessibility of the relevant cysteine residues. The inter-heavy chain disulfides in IgG lie in the disordered hinge region (Harris *et al.*, 1999), whereas in IgE-Fc they are rather more occluded between the Cε2 domains (Wan *et al.*, 2002). Consistent with this observation, the interchain disulfide of an IgE-Fc (Cε3-4) fragment (Shi *et al.*, 1997) containing C328, but lacking any such obstruction by Cε2 domains, was found to be reduced in ~0.5mM DTT (J. Hunt, 2004, PhD Thesis).

In IgY, interchain disulfides putatively occur between homologous residues in Cυ2 and additionally in Cυ3 (Parvari *et al.*, 1988). The minimum concentration of DTT required to reduce inter-heavy chain disulfides was determined for chicken IgY and (to ensure comparability) reproduced for human IgG1 and IgE-Fc using the same methodology and reagents (Figure 5.4.6 A, B & C). The order of susceptibility to reduction was found to be IgG1>IgY>IgE. An approximately ten fold higher concentration of DTT was required to reduce all of the inter-heavy chain bonds in IgY than IgG1, and ten fold higher again to reduce those of IgE-Fc. This suggests that the bonds are considerably less accessible in IgY than IgG1, but not to the same extent as in IgE. The structure of IgY in this region would therefore appear to be distinct from both mammalian isotypes, although greater similarity to IgE than IgG may be inferred, consistent

with the locations of two of the cysteine pairs, which are conserved between IgY and IgE, but not with IgG.

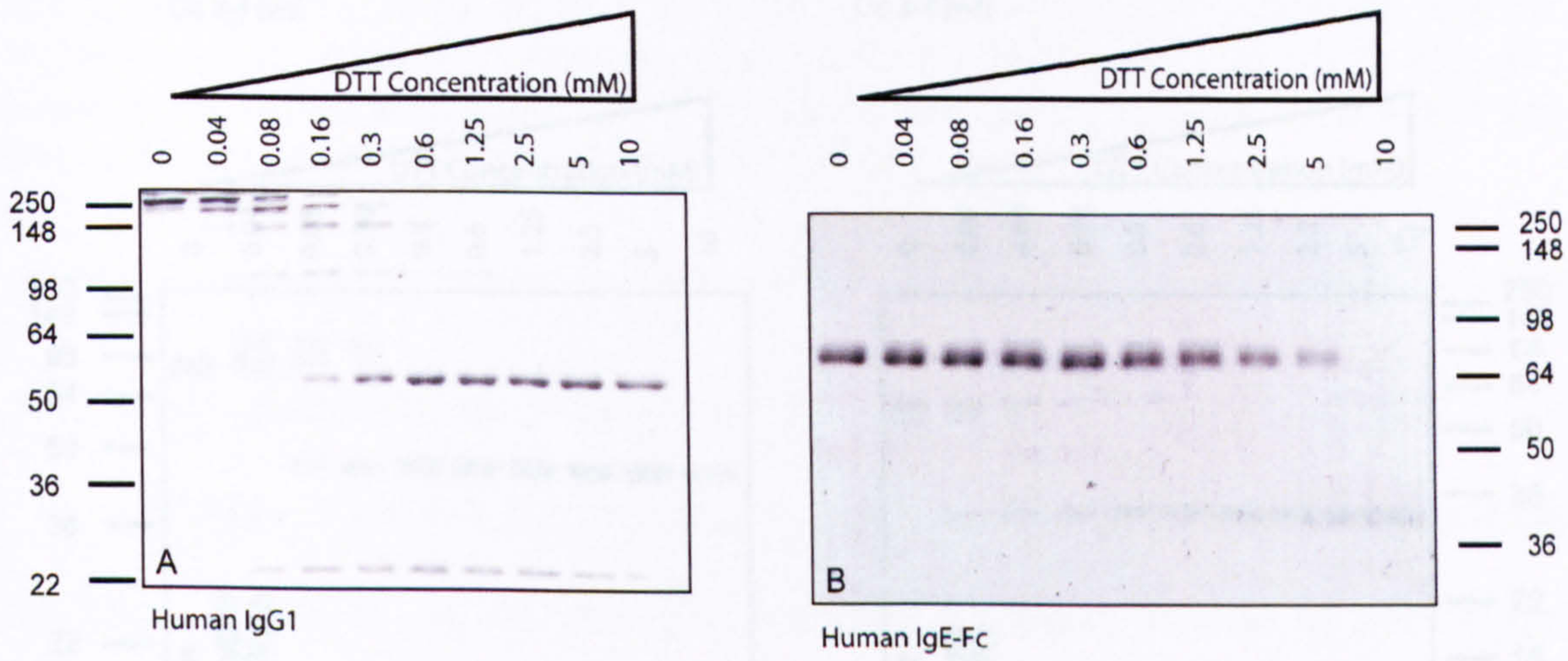
5.4.6.1 Determination of sensitivity of IgY-Fc interchain disulphide bonds to reduction by DTT

The possibility of differential susceptibility to DTT reduction of the IgY inter-heavy chain disulfide bonds was investigated in the context of IgY-Fc (Cυ2-4 and Cυ3-4) fragments. Slightly lower concentrations of DTT were found to reduce the interchain disulfides in the Cυ2-4 [wt] Fc fragment (~0.5mM) than in the whole antibody (~1mM) (Figure 5.4.6 C & D). This could indicate that domains in Fab region (such as Cυ1) contribute to the reduced accessibility observed in the complete IgY, either directly or by altering the orientation of the Cυ2 domains.

Significantly, the Cυ2-4 [N308Q N407Q] mutant was reduced by a more than four-fold lower DTT concentration than the wild type Cυ2-4, whereas the [N308Q] mutant required the same higher concentration (Figure 5.4.6 D, E & F). The removal of glycosylation at N407 therefore appears to influence the accessibility of the interchain disulfides, which further supports an important role in maintenance of quaternary structure for oligosaccharide at this position.

The sensitivity of the non-conserved 'extra' C347 disulfide was determined in the context of a Cυ3-4 fragment. Cυ3-4 fragments disulfide bonded at either C340, C347 or both were all fully reduced by ~0.3mM DTT. Comparison with the results obtained with whole IgY is difficult as these fragments lack Cυ2, although it can be surmised that the disulfide at C347 has the same accessibility as that at C340. The residues around C347 are homologous to

those in IgE which form the binding site for FcεRIα (Garman *et al.*, 2000), as discussed in chapter 3, so it is interesting that they appear to be readily accessible in Cυ3.



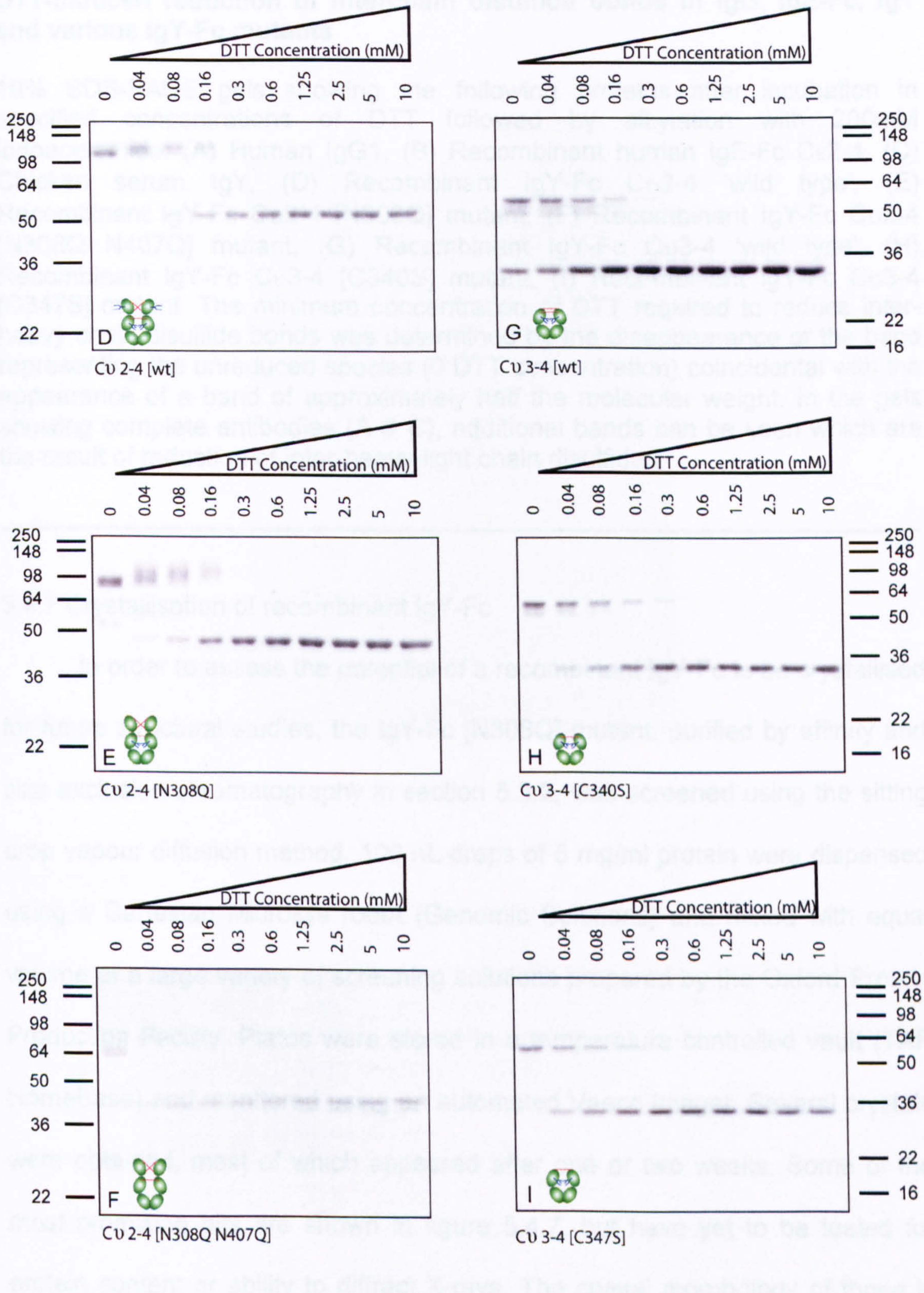

DTT Concentration (mM)
0
0.04
0.08
0.16
0.3
0.6
1.25
2.5
5
10
250
148
98
64
50
36
22
D
Cυ 2-4 [wt]
G
Cυ 3-4 [wt]
E
Cυ 2-4 [N308Q]
H
Cυ 3-4 [C340S]
F
Cυ 2-4 [N308Q N407Q]
I
Cυ 3-4 [C347S]
16

Figure 5.4.6 (previous two pages)
DTT-induced reduction of interchain disulfide bonds in IgG, IgE-Fc, IgY and various IgY-Fc mutants

10% SDS-PAGE gels showing the following proteins after incubation in specified concentrations of DTT followed by alkylation with 200mM iodoacetamide: (A) Human IgG1, (B) Recombinant human IgE-Fc Cε2-4, (C) Chicken serum IgY, (D) Recombinant IgY-Fc Cυ2-4 'wild type', (E) Recombinant IgY-Fc Cυ2-4 [N308Q] mutant, (F) Recombinant IgY-Fc Cυ2-4 [N308Q N407Q] mutant, (G) Recombinant IgY-Fc Cυ3-4 'wild type', (H) Recombinant IgY-Fc Cυ3-4 [C340S] mutant, (I) Recombinant IgY-Fc Cυ3-4 [C347S] mutant. The minimum concentration of DTT required to reduce inter-heavy chain disulfide bonds was determined by the disappearance of the band representing the unreduced species (0 DTT concentration) coincidental with the appearance of a band of approximately half the molecular weight. In the gels showing complete antibodies (A & C), additional bands can be seen which are the result of reduction of inter-heavy-light chain disulfides.

5.4.7 Crystallisation of recombinant IgY-Fc

In order to assess the potential of a recombinant IgY-Fc to be crystallised for future structural studies, the IgY-Fc [N308Q] mutant, purified by affinity and size exclusion chromatography in section 5.3.2, was screened using the sitting drop vapour diffusion method. 100 nL drops of 5 mg/ml protein were dispensed using a Cartesian Microsys robot (Genomic Solutions) and mixed with equal volume of a large variety of screening solutions prepared by the Oxford Protein Production Facility. Plates were stored in a temperature controlled vault (TAP HomeBase) and monitored using an automated Veeco imager. Several crystals were obtained, most of which appeared after one or two weeks. Some of the most promising hits are shown in figure 5.4.7, but have yet to be tested for protein content or ability to diffract X-rays. The crystal morphology of those in figure 5.4.7 B were very similar to crystals of papain-derived IgY-Fc (Figure 3.5 B), which could imply a similar mode of molecular packing.

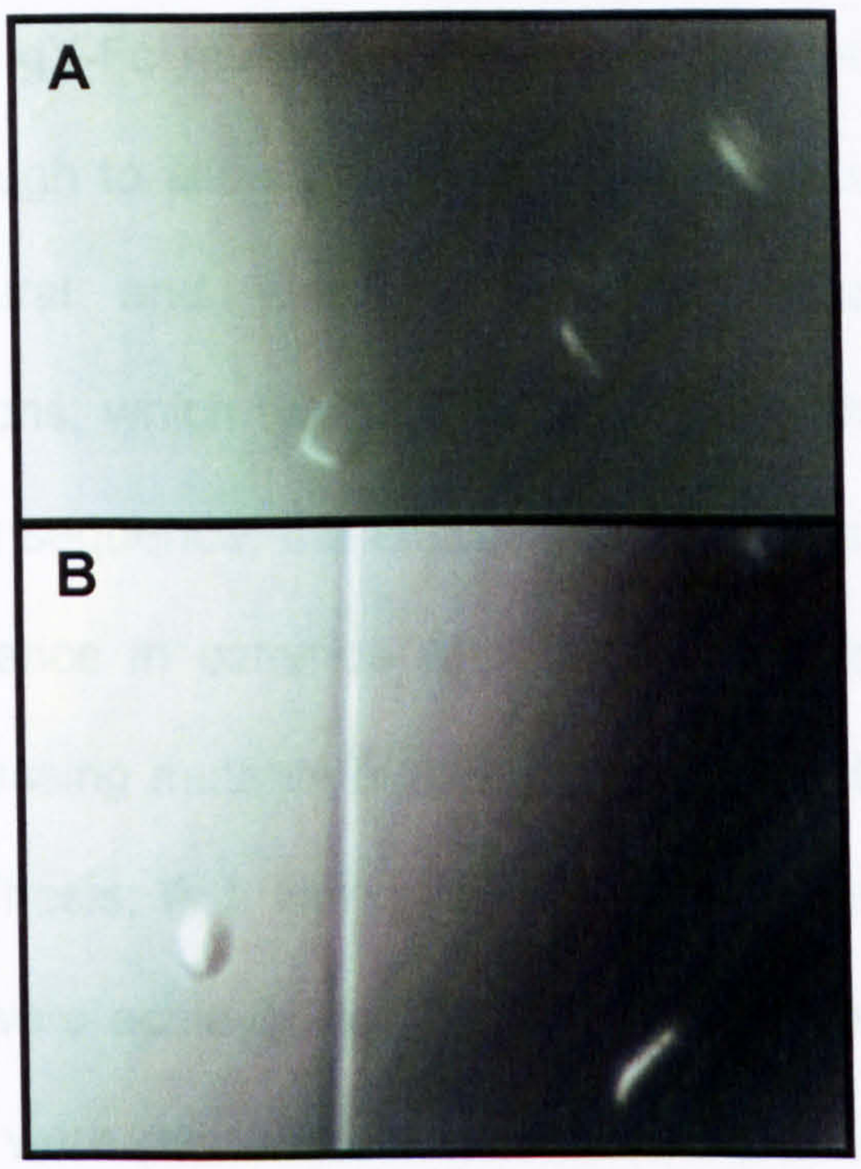


Figure 5.4.7
Initial hits in an IgY-Fc crystallisation screen

Crystals obtained in a sitting drop vapour diffusion screen of recombinant IgY-Fc Cυ2-4 [N308Q]. Screening conditions were (A) 0.1M Tris pH 8.5 + 6M Ammonium Nitrate, (B) 0.8M Ammonium Sulphate + 0.1M MES pH 6.0. Images were obtained using a Veeco automated plate imager.

5.5 Discussion

In this chapter, the mammalian expression vectors designed and constructed in chapter 4 were used to create a series of stable cell lines which secrete recombinant IgY-Fc mutants. Cells were selected so that expression levels were high enough to allow practical quantities of purified material to be obtained for structural and functional studies, including screening for crystallisation conditions, which typically requires milligrams of protein. Except for the IgY-Fc coding sequence, the expression vectors had been designed to have as much sequence in common with vectors previously used to create stable cell lines expressing mutants of IgE-Fc to around 100mg/L or more (R.J. Young, 1994, PhD Thesis; A.J. Henry, 1996, PhD Thesis). As hoped, similar levels of production were achieved for four of the seven mutants (94-340mg/L). The remaining three were produced in lower levels (16-48mg/L), although this was still sufficient for initial analysis, so screening for higher producers was not performed.

Material was purified to a sufficient standard to allow experimentation to determine various structural characteristics and to confirm whether the intended modifications to each IgY-Fc mutant have indeed been made. Molecular weights were determined using a combination of native and non-native techniques; size exclusion chromatography, DLS (not shown) and SDS-PAGE (reducing and non-reducing). The results for all of the Fc mutants were consistent with disulfide-linked homodimers of the expected size (containing either four or six Ig domains), although a substantial proportion of the Cυ3-4 [wt] preparation appeared to be monomeric in SDS even in the absence of reducing agents, indicating that interchain disulfides had not formed. This suggests that

the presence of a complete Cυ2 is necessary for the efficient formation of interchain disulfides. A scheme for the artefactual formation of a C340-C347 intrachain bond as the protein is folding may be envisaged (Figure 5.5), which the presence of a folding or folded Cυ2 would prevent. Mutation of one of these cysteines to serine precludes the problem and an intrachain bond is formed. The Cυ3-4 sequence was engineered to include some residues from Cυ2 in order to be homologous to the recombinant IgE Cε3-4 fragment used in previous studies, which includes C328 (from Cε2) so that disulfide linked homodimers form (Hunt *et al.*, 2005; McDonnell *et al.*, 2001; Shi *et al.*, 1997). Whilst it would have been interesting to study the effect of two interchain disulfides in a Cυ3-4 fragment, confirmation of the presence of a disulfide at C347 makes the inclusion of C340 unnecessary to achieve a dimeric fragment. Therefore, the IgY-Fc mutant most similar to the Cε3-4 fragment is actually Cυ3-4 [C340S].

The evidence for the existence of an inter-heavy chain disulfide at C347, potentially an 'extra' bond not present in IgE, was previously limited to observations made with papain-derived Cυ3-4 Fc fragments (see chapter 3). Although a C347 interchain disulfide was inferred by the dimerisation of the Cυ3-4 [C340S] mutant, this does not establish whether the same bond is present in the whole IgY molecule. To address this, an Ellman assay of IgY and Cυ2-4 [wt] was performed. The assay showed that no free sulfhydryls were present in the whole molecule or the Fc, which confirms that all cysteines are indeed involved in disulfide bonds.

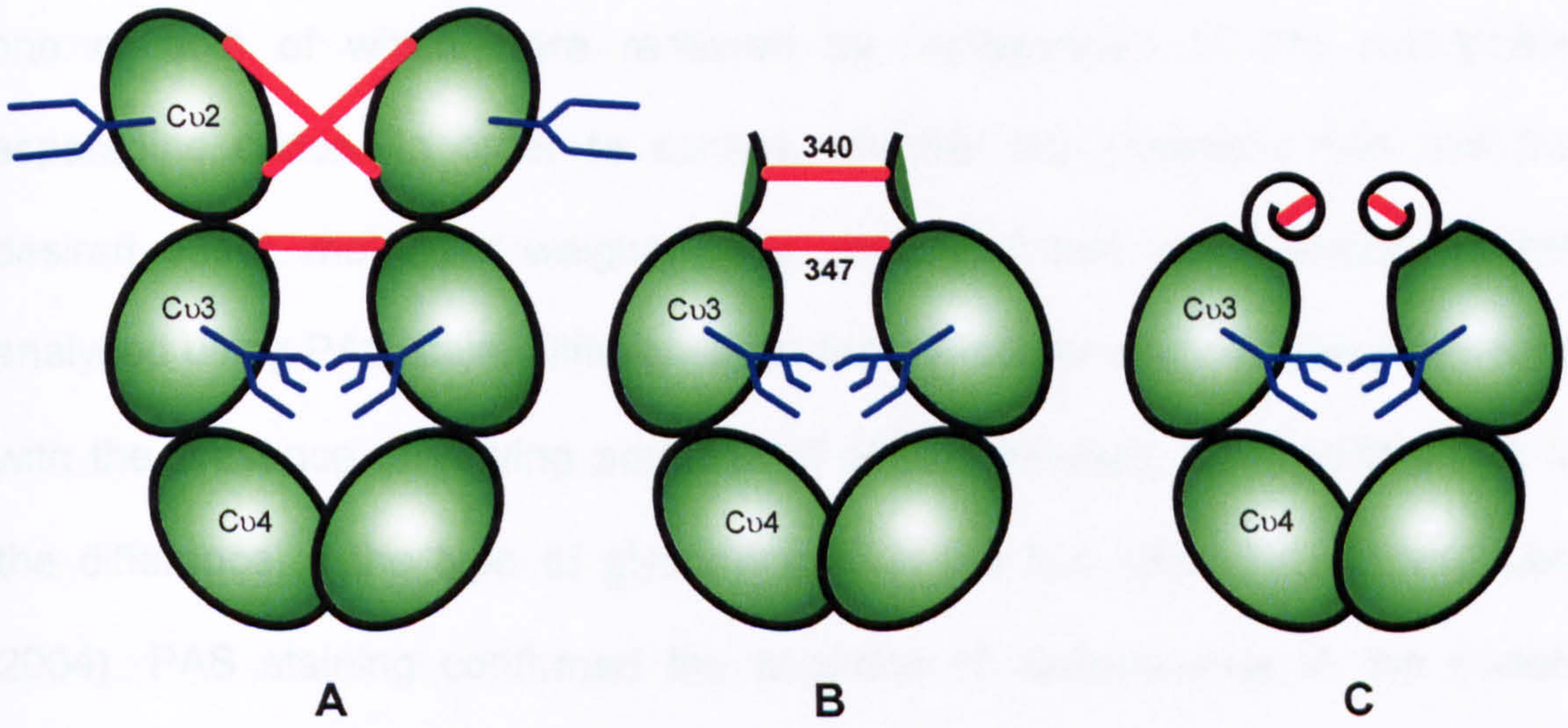


Figure 5.5
Erroneous disulfide pairing in IgY-Fc Cυ3-4 [wt]

Schematic showing a possible explanation for the presence of fragments lacking interchain disulfide bonds in mammalian expressed Cυ3-4 [wt]. In Cυ2-4 (A) disulfide bonds may form as shown, with C340 paired in a crossed fashion with C252, as occurs between the homologous pairs in Cε2 of IgE (Wan *et al.*, 2002). When the Cυ3-4 [wt] fragment was designed, a fragment with parallel disulfides was expected (B), although at least 50% of expressed material was found to contain only non-covalent interactions (shown between Cυ4 pairs). This could be the result of misfolding and formation of an intradomain disulfide bond between C340 and C347 (C). An alternative explanation may be the formation of free sulfhydryls, although this seems unlikely as removal of one or the other cysteine results in a disulfide linked dimer.

The Cυ2-4 [wt], [N308Q] and [N308Q N407Q] mutants were designed to differ in glycosylation state in order to study the structural and functional effects of N-linked carbohydrate. IgY-Fc is predicted to have two glycosylation sites, one or both of which were removed by mutagenesis of the appropriate asparagine codon. In order to confirm whether the mutations had had the desired effect, molecular weights were compared and carbohydrate content analysed using PAS stain. Differences in the molecular weights were consistent with the presence of varying amounts of oligosaccharide, as expected due to the difference in the type of glycosylation at the two sites (Suzuki and Lee, 2004). PAS staining confirmed the absence of carbohydrate in the mutant lacking both glycosylation sites, Cυ2-4 [N308Q N407Q]. The significance of the production of this fragment will be discussed further in chapter 7.

Once the presence of the intended differences between the mutants had been examined, some preliminary studies into their structural properties were performed. A rough comparison of melting temperatures made using DLS revealed that the role of glycosylation and disulfide linkage in the maintenance of thermal stability appears to be well conserved between IgG, IgE and IgY. Overall, however, the melting temperature of IgY was found to be more similar to IgG than IgE, which could indicate that the instability of Cε3 in IgE is not a feature of the homologous Cυ3 domains in IgY. This may imply a significant difference between their Fc receptor interactions as this property of Cε3 has been suggested to represent a means of fine tuning the (high) affinity and specificity of the interaction with FcεRI (Price *et al.*, 2005). However, other investigators have noted that chicken IgY is more sensitive than cow, goat and pig IgG to pH- or guanidine-induced denaturation (Shimizu *et al.*, 1993). Further

experiments are therefore required to better establish the stability of IgY-Fc. The cloning and expression of individual Cυ3 domains would allow emulation of the experiments performed with Cε3 by Price *et al.* (2005), particularly ANS dye binding (which can be used to confirm the 'molten globule' state), although soluble IgY-Fc receptor would be required for characterisation of changes occurring upon binding using NMR spectroscopy (Harwood *et al.*, 2006).

Ultimately, an important question to answer is whether IgY shares the acutely bent, asymmetrical conformation of IgE in its unbound state (Wan *et al.*, 2002). Differences in the accessibility of the inter-heavy chain disulfides in IgY and IgE (determined by susceptibility to reduction with DTT) suggests that their structures are not identical. The bend in unbound IgE can be observed using electron microscopy and is consistent with X-ray and neutron scattering data in solution (B.J. Sutton and A.J. Beavil, personal communication). Noll *et al.* noted that IgY bound to ribosomes (via their Fab regions) had an IgG-like structure (Noll *et al.*, 1982), although IgY has been suggested to alter the conformation of its Fc upon binding to antigens, due to the exposure of Staphylococcal protein A reactive sites (Barkas and Watson, 1979). Determination of the crystal structure of IgY-Fc using X-ray diffraction data would provide an answer to this question and allow a far more detailed comparison of structural differences between the Fc fragments of IgY, IgG and IgE to be made. Crystal structures of each of the mutants would also afford insights into nature of the structural contributions of glycosylation and disulfide linkage.

In order to asses whether IgY undergoes a conformational change upon receptor binding, as IgE is hypothesised to do in order to facilitate its distinctive kinetics, a complex of IgY-Fc bound to a soluble form of Fc receptor could be

crystallised, as has been achieved for IgE-Fc (Cε3-4) and FcεRI (Garman *et al.*, 2000). As a soluble IgY-Fc receptor has yet to be produced, this is not yet possible. Alternatively, a system to visualise the conformational change in IgE-Fc has been developed (J. Hunt and A. J. Beavil, personal communication) which could be adapted easily for IgY-Fc and used with IgY-Fc receptor-bearing cells (see chapter 6) rather than soluble receptor. Briefly, the technique relies on the abrogation of a Fluorescence (Förster) Resonance Energy Transfer (FRET) signal between donor and acceptor fluorophores linked to the N- and C-termini of each heavy chain Fc. Unbound, the pair are within the molecular distance required for FRET, but they move too far apart for energy transfer upon binding. It would be interesting to discover whether the same behaviour is observed with IgY-Fc.

Chapter 6

Search for IgY Fc Receptors and Cloning of the First Avian Fc Receptor Family Member, chFcR/L

6.1 Overview

In order to study the kinetics of the interaction between antibodies and their Fc receptors (FcR) using modern biophysical techniques such as Surface Plasmon Resonance (SPR) and Analytical Ultracentrifugation (AUC), soluble receptor and ligand is required (Hunt *et al.*, 2005; Paetz *et al.*, 2005).

The previous chapters detail the production of recombinant soluble IgY-Fc and various mutants. The present chapter describes attempts to identify and isolate receptors for IgY-Fc, which are functionally equivalent to the mammalian FcR for IgG, and/or IgE, with the aim of allowing recombinant soluble forms to be produced in future studies.

How IgY is apparently able to mediate functions attributed specifically to IgG or IgE in mammals is currently unknown. Although IgY is found in several vertebrate classes, at the time of this investigation the only IgY receptor identified is exclusively expressed on the chicken yolk sac (West *et al.*, 2004); no known leukocyte IgY-Fc receptors have been sequenced. There is ample functional evidence to confirm the existence of such receptors; various avian immune cell effector responses are triggered by recognition of IgY-coated pathogens and IgY can be used to passively sensitise avian tissues (Chand *et*

al., 1976; Faith and Clem, 1973; Kogut *et al.*, 2001; Qureshi *et al.*, 2000). In mammals, these mechanisms involve FcγRs and FcεRs.

In addition to the chicken yolk sac receptor, two non-mammalian Fc receptors have been characterised, both of which recognise non-IgY isotypes: a receptor for IgM in the channel catfish, IpFcR (Stafford *et al.*, 2006) and a receptor for secretory IgA in the chicken, GG-pIgR (Wieland *et al.*, 2004). Interestingly, all three of these receptors have functional analogues in mammals, but only GG-pIgR is also homologous to its mammalian counterpart. Although FcRY provides a means of maternal antibody transfer to the developing chick, just as FcRn does for embryonic mammals (Matre *et al.*, 1981), the receptors are actually members of non-homologous protein families (West *et al.*, 2004).

Convergent evolution of sequences, where unrelated proteins are selected to perform identical functions, dictates caution when formulating strategies to identify them using certain techniques. The first approach was to exploit the affinity and specificity of receptor for ligand by attempting to purify complexes of IgY bound to its receptor from solubilized cell membranes. This method was used to successfully isolate FcεRI, the high affinity receptor for IgE (Conrad and Froese, 1978). The second approach involved data mining of the recently released chicken genome (Hillier *et al.*, 2004) and expressed sequence tag (EST) databases for potential Fc receptor sequences. This method relies on sequence similarity with mammalian FcRs to identify chicken orthologues. As outlined above, this assumption could lead to failure to spot receptors with little or no homology. This problem would not apply to the protein purification strategy. Genome/EST analysis is nevertheless worthwhile, as some receptors

can prove particularly recalcitrant to isolation using protein methods. Mouse FcγRIV was recently identified using this approach (Nimmerjahn *et al.*, 2005).

6.2 Affinity purification of avian IgY-Fc receptors analogous to mammalian FcγR and FcεR

6.2.1 Indirect evidence for an IgY-Fc receptor on chicken basophils

Whilst some facets of the immune system appear to be absent or markedly different in birds, many key features are well conserved with mammals. Indeed, much of our early understanding of human cellular immunity was derived from parallels with the chicken system. In mammals, the high affinity receptor for IgE, FcεRI, is found in greatest numbers on mast cells and basophils - several hundred thousand molecules per cell (Conrad *et al.*, 1975; Conroy *et al.*, 1977; Ishizaka *et al.*, 1973). Historically, a rat basophilic leukaemia (RBL) cell line was used to isolate FcεRI protein (Conrad *et al.*, 1976) as these cell types are found in relatively low numbers *in vivo*.

In birds, Th1/Th2 polarisation has been demonstrated (Degen *et al.*, 2005). The repertoire of bird white blood cells and immune chemistry is also known to include basophilic granulocytes and homologues of many of the key T2/Th2 cytokines, which in mammals regulate the branch of the immune system involving these effector cells (Avery *et al.*, 2004). The lack of a functional IL-5 gene led some researchers to speculate that certain types of granulocyte may be non-functional in the chicken (Kaiser *et al.*, 2005), although a protein with some similarity to IL-5, KK34, may perform the same role (Koskela *et al.*, 2004). The presence of basophil infiltrates in avian anaphylactic responses (Carlson

and Allen, 1969; Chand *et al.*, 1976) and the potential for basophils to be passively sensitized (Wilson and Heller, 1976) suggest that bird basophils are important effector cells in antibody-mediated inflammatory reactions. It therefore seemed probable that chicken basophils were strong *a priori* candidates for the presence of IgY-Fc receptors.

Cell sorting was used to establish which cell type in chicken peripheral blood possessed the highest quantity of IgY-Fc receptors per cell. Briefly, buffy coat layer cells were isolated, stained with fluorescently labelled anti-IgY antibodies and separated on the basis of fluorescence intensity using a FACS Aria cell sorter. The experiment assumed that the cell population with the highest fluorescence intensity had the greatest number of anti-IgY antibodies bound, and thus the greatest number of IgY molecules ostensibly bound to Fc receptors. The sorted population were stained with Giemsa and identified as predominantly basophils (Figure 6.2.1). Attempts were then made to isolate peripheral blood basophils in a more practical way, in order to obtain sufficient quantities for further studies.

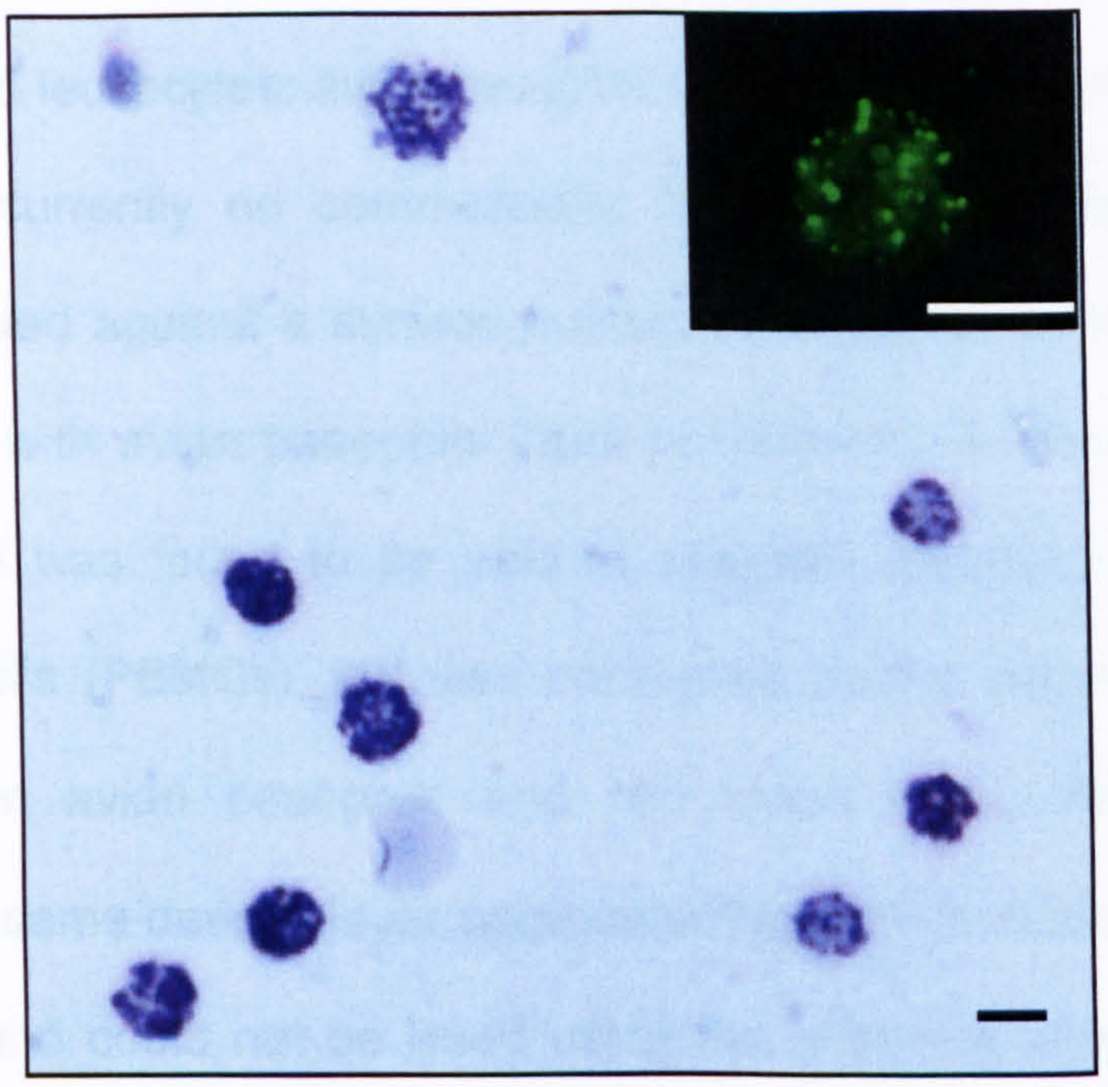


Figure 6.2.1
Basophils FACS sorted from chicken peripheral blood on the basis of bound IgY

Chicken buffy coat layer cells displaying the brightest fluorescence when stained with anti-IgY-FITC antibodies (inset) were sorted using a FACS Aria (BD Biosciences). Cells were mounted on slides using a cytospin centrifuge (Shandon) and stained with Giemsa (main field). Darkly staining granules and multi-lobed nuclei are characteristic of basophils. Scale bar = 10μm (main field and inset).

Several methods are well established for the purification of basophils from human peripheral blood. Erythrocytes can be removed easily by lysis in hypotonic solutions, to which white cells are less susceptible. Basophils can be enriched by adsorption onto magnetic beads using antibodies specific for surface markers or by depleting all other leukocytes in the same manner (Bjerke *et al.*, 1993; Haisch *et al.*, 1999). Alternatively, density gradient centrifugation exploits the difference in density between leukocytes to separate them in layers of a liquid phase such as sucrose (Kepley *et al.*, 1994).

Unfortunately, these methods were found to be problematic for separation of bird leukocytes: avian basophil surface markers are poorly studied and there are currently no commercially available anti-basophil antibodies. Antibodies directed against a surface marker of human basophils, CD123, did not cross react with avian basophils (data not shown). A Percoll (Amersham) density gradient was found to be able to separate chicken peripheral blood mononuclear cells (PBMCs), but was hampered by the apparent similarity in density between avian basophils and red blood cells, which consistently migrated to the same density layer upon centrifugation. In birds, red blood cells are nucleated and could not be lysed using the methods usually employed to prepare white blood cells from mammalian blood. Centrifugation to separate the buffy coat layer, which contains most white cells, prior to density gradient separation was found to be the most effective means of preparing avian basophils to ~70-80% purity. However, this method was found to be impractical for purification of the quantities of cells that would be required for more detailed studies.

In order to perform cell binding studies, acidic conditions can be used to strip endogenously bound IgE from human basophils (Pruzansky *et al.*, 1983). The use of both lactic acid and glycine was investigated for the removal of IgY from chicken basophils, but this method failed to remove a satisfactory quantity without severely damaging the cells (data not shown).

A chicken basophil cell line adapted to IgY-free medium would be the most obvious solution to the problem of receptor occupancy and would, in principle, provide an unlimited number of cells without laborious purification procedures from chicken peripheral blood. Unfortunately, only one avian

basophil-like cell line has been reported in the literature (Moscovici *et al.*, 1989), stocks of which no longer exist (G. Moscovici, personal communication).

6.2.2 Identification of a chicken monocyte Fc receptor

Monocytes in both birds and mammals are important blood-borne leukocytes, which phagocytose antibody-coated pathogens and mediate antibody-dependent cellular cytotoxicity (ADCC) of infected or cancerous cells. An IgY-Fc receptor analogous to the high affinity receptor for IgG, FcγRI, may therefore be present on chicken monocytes.

Different values appear in the literature for the number of FcγRI molecules per cell on human monocytes: 4,300 ± 1,304 (Karagiannis *et al.*, 2003), 45,000 ± 10,700 (Jungi and Hafner, 1986). Mouse monocytes appear to express much higher levels, ~100,000 FcγRI per cell (Unkeless and Eisen, 1975). It was therefore difficult to predict how many IgY receptors should be expected on avian monocytes, which proved to be a crucial determinant of the practicability of certain protein methods.

A chicken monocyte cell line, MQ-NCSU, for which functional evidence for IgY-mediated phagocytic activity has been demonstrated (Qureshi *et al.*, 1990), was used to confirm the existence of Fc receptors for IgY using flow cytometry (Figure 6.2.2). MQ-NCSU cells were adapted for growth in media containing foetal bovine serum (FBS), but not chicken serum, so IgY would not be present to occupy receptors. Cells were able to bind IgY (Figure 6.2.2 B & C) even in the presence of a 400-fold excess of IgY-Fab fragments (Figure 6.2.2 D), confirming that binding is dependent upon the presence of IgY-Fc.

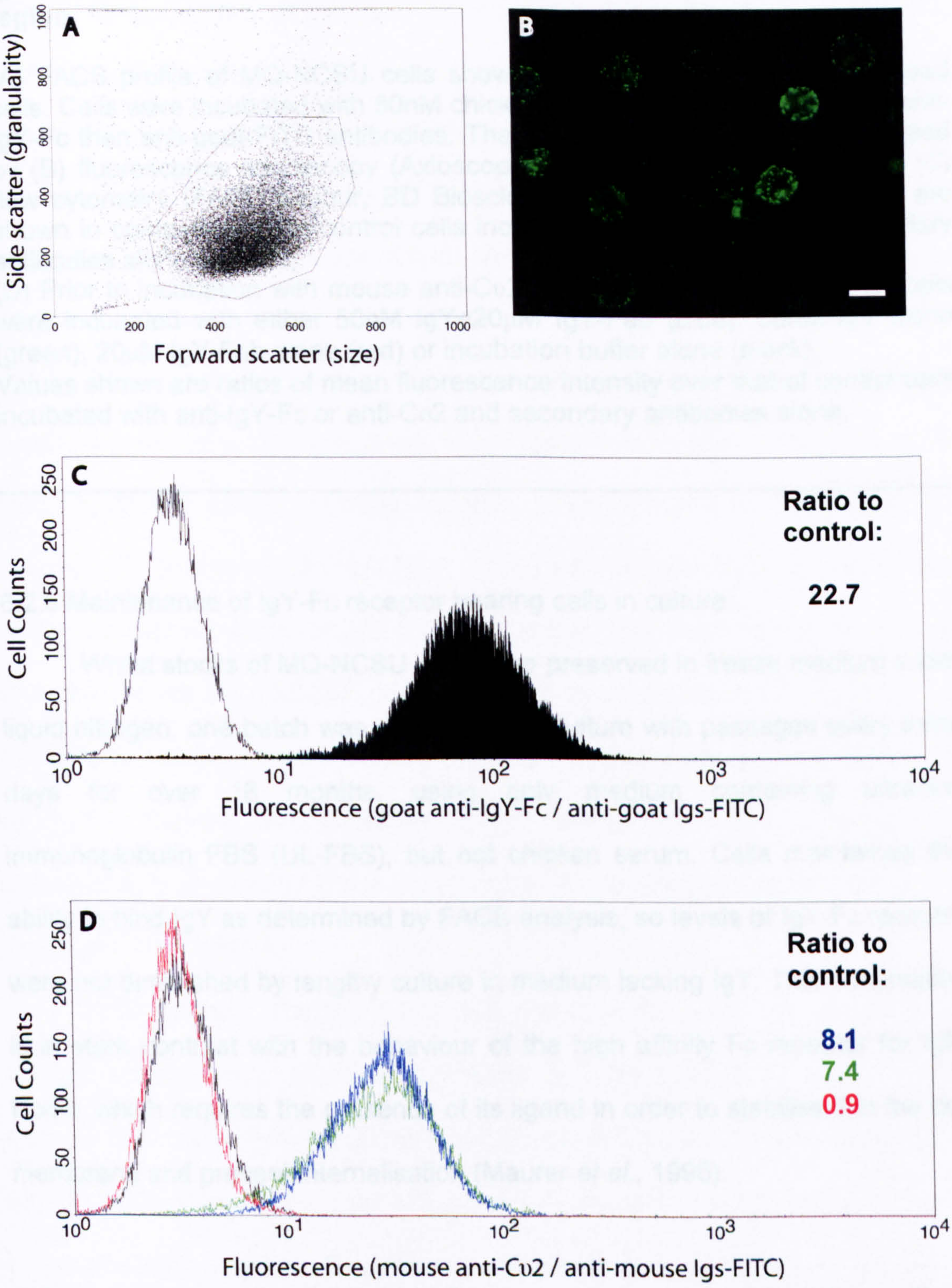

A
Side scatter (granularity)
0
200
400
600
800
1000
Forward scatter (size)
B
C
Cell Counts
0
50
100
150
200
250
10^0
10^1
10^2
10^3
10^4
Ratio to control:
22.7
Fluorescence (goat anti-IgY-Fc / anti-goat Igs-FITC)
D
Cell Counts
Ratio to control:
8.1
7.4
0.9
Fluorescence (mouse anti-Cυ2 / anti-mouse Igs-FITC)

Figure 6.2.2 (previous page)
IgY binds to MQ-NCSU chicken monocytes and is dependent on the Fc region

(A) FACS profile of MQ-NCSU cells showing the gate used to exclude dead cells. Cells were incubated with 50nM chicken serum IgY, followed by goat anti-IgY-Fc then anti-goat-FITC antibodies. The cells were then fixed and analysed by (B) fluorescence microscopy (Axioscope, Zeiss, Scale bar = 10μm) or (C) flow cytometry (FACS Calibur, BD Biosciences). IgY coated cells (filled) are shown in comparison with control cells incubated with anti-IgY-Fc / secondary antibodies alone (unfilled).
(D) Prior to incubation with mouse anti-Cυ2 followed by anti-mouse-FITC, cells were incubated with either 50nM IgY+20μM IgY-Fab (blue), 50nM IgY alone (green), 20μM IgY-Fab alone (red) or incubation buffer alone (black).
Values shown are ratios of mean fluorescence intensity over that of control cells incubated with anti-IgY-Fc or anti-Cυ2 and secondary antibodies alone.

6.2.3 Maintenance of IgY-Fc receptor bearing cells in culture

Whilst stocks of MQ-NCSU cells were preserved in freeze medium under liquid nitrogen, one batch was maintained in culture with passages every three days for over 18 months, using only medium containing ultra-low immunoglobulin FBS (UL-FBS), but not chicken serum. Cells maintained the ability to bind IgY as determined by FACS analysis, so levels of IgY-Fc receptor were not diminished by lengthy culture in medium lacking IgY. This observation is in stark contrast with the behaviour of the high affinity Fc receptor for IgE, FcεRI, which requires the presence of its ligand in order to stabilise it in the cell membrane and prevent internalisation (Maurer *et al.*, 1996).

6.2.4 Attempts to purify IgY-Fc receptor complexes using affinity chromatography

Previous attempts have been made to isolate IgY receptors from chicken monocytes using immobilised IgY, but these were unsuccessful and

inconclusive (Morkowski and Nowak, 1981). A similar method did prove to be suitable for preparation of yolk sac IgY receptors, however (West *et al.*, 2004), and so a modified version was investigated for purification of receptors from MQ-NCSU cells. Briefly, cell membrane proteins from MQ-NCSU cells pre-incubated in saturating levels of IgY were solublised using a non-ionic surfactant, NP-40, which has been shown to allow solublisation without disruption of ligand:receptor (and receptor subunit) interactions (Conrad *et al.*, 1976). Preparations were then incubated with anti-IgY coupled to agarose beads in order to purify complexes of IgY bound to receptor. The quantity of cells required to obtain sufficient material for visualisation on SDS-PAGE gel stained with Coomassie Brilliant Blue (~1µg) was calculated using an estimate of the receptor molecular weight (50 kDa) and an approximation of the number of receptors per cell (10,000), as determined by quantitation of bound IgY by Western blot of cell lysates, assuming 1:1 stoichiometry (experiment performed by Dr. R.A. Calvert, Randall Division, King's College London). A more accurate determination of receptor number per cell was subsequently performed and is discussed in chapter 7.

Unfortunately, despite numerous attempts to solve the problem by varying agarose bead washing conditions, a large number of proteins were eluted which could be ruled out as receptor candidates due to their presence in control preparations lacking IgY (Figure 6.2.4). Several faint bands can be seen in the eluate from IgY positive cell preparations (Figure 6.2.4 lane 4). A band of roughly 55kDa from a larger preparation was transferred onto PVDF membrane for N-terminal sequencing (performed by Dr. J.N. Keen, University of Leeds). An accurate sequence could not be obtained due to the presence of multiple

proteins, despite the lack of any other clearly defined bands on the excised strip of PVDF when stained with Ponceau Red.

The presence of so many bands in the negative control casts doubt on the feasibility of the technique. As there is currently no structural information about the receptor, it is very difficult to judge which bands are any more likely to be a receptor than others. For example, the mammalian high affinity receptor for IgE, FcεRIα, appears as a diffuse smear on SDS-PAGE gels due to multiple N-glycosylation sites, which highlights the further problem that an IgY-Fc receptor may not necessarily appear as a tightly resolved band. For practical reasons, other approaches to IgY-Fc receptor characterisation were pursued.

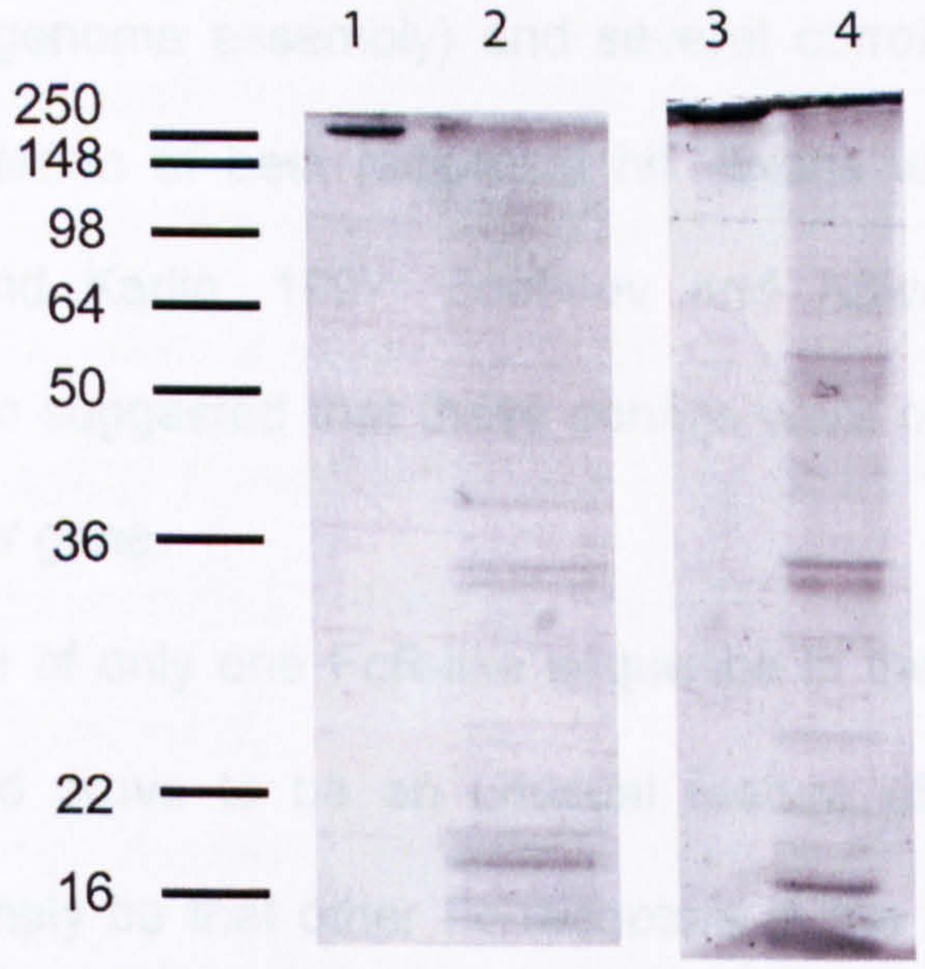


Figure 6.2.4
Attempted affinity purification of IgY receptor from MQ-NCSU cells

12% SDS-PAGE gel stained with Coomassie showing IgY (lanes 1 & 3) and purified eluate from anti-IgY agarose beads incubated with MQ-NCSU solublised total cell protein prepared from cells incubated in buffer alone followed by washing (lane 2) or IgY followed by washing (lane 4).

6.3 Identification and cloning of a chicken Fc receptor homologue

The following section details a bioinformatics-based approach to the search for IgY-Fc receptors. Whilst not without its limitations (as outlined in section 6.1), the strategy has nevertheless yielded some interesting results, some of which have subsequently been published (Taylor *et al.*, 2007) and confirmed by other investigators (Fayngerts *et al.*, 2007).

6.3.1 Data mining of chicken genome and EST libraries

Mammalian Fc receptor (FcR) sequences were used with the Basic Local Alignment Search Tool (BLAST) to search the chicken (*Gallus gallus*) genome (http://www.ensembl.org/Gallus_gallus/index.html) and BBSRC chickEST database (http://www.chick.umist.ac.uk/). Two contigs (7652.1 and 3134.3 in the February 2004 genome assembly) and several corroborating ESTs were identified by the criterion of best reciprocal hit. Exons were predicted using Genscan (Burge and Karlin, 1997; Zdobnov and Apweiler, 2001). Partial sequence duplication suggested that these contigs were overlapping, covering four exons of a larger gene.

The presence of only one FcR-like sequence in the chicken genome is surprising and could prove to be an unusual feature of avian immunology. However, it may simply be that other Fc receptors in the bird are unrelated to mammalian analogues or that the genome and EST libraries are incomplete. Indeed, many immune-related families that consist of numerous closely related sequences (such as the LRC and MHC) have proven difficult to fully sequence by the shotgun approach used for the chicken genome (Burt, 2005).

6.3.2 PCR cloning of a chicken Fc receptor homologue, chFcR/L

Primers specific for exons identified in the sequences were designed and used in 5' and 3' RACE PCR reactions (see chapter 2) on cDNA derived from a splenocyte cDNA library (a gift from Dr. J. Young, Institute for Animal Health). Sequences were aligned in ChromasPro to produce a contiguous sequence for the full-length transcript.

6.3.3 Analysis of chFcR/L coding sequence (CDS)

The full-length cDNA was translated using the ExPasy Translate tool (Swiss Institute of Bioinformatics) and the amino acid sequence analysed using several software packages. The cDNA contained a 1,308bp CDS (Figure 6.3.3 i), which putatively encoded a 19 amino acid (aa) signal peptide, predicted using SignalP3.0, http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen *et al.*, 2004), and a novel 416aa transmembrane glycoprotein consisting of four extracellular domains that belong to the immunoglobulin (Ig) superfamily, a transmembrane helix, and a short cytoplasmic tail were identified using InterProScan (Burge and Karlin, 1997; Zdobnov and Apweiler, 2001). The protein was termed chFcR/L, adapting recently established terminology guidelines (Maltais *et al.*, 2006).

The amino acid sequence of chFcR/L was used to BLAST search the NCBI non-redundant protein database, which confirmed that the protein is homologous to many important immunological players (Table 6.3.3 ii): the mammalian Fc receptor (FcR) family, including the high affinity receptors for both IgG and IgE, and the IgM-binding catfish Fc receptor (Stafford *et al.*, 2006), as well as several potential FcR-related sequences from *Xenopus* (Guselnikov

et al., 2004). The search also identified several members of a family of proteins recently discovered in mouse and human, which genomic proximity and homology to the FcR classifies as 'Fc receptor-like' (FCRL). Although they possess Ig domains closely related to those responsible for Fc binding in FcγRI-IV and FcεRI, the FCRL appear to lack the ability to bind any Fc isotype (Davis *et al.*, 2002). As yet, no ligands have been found, but the preferential expression of FCRLs on B cells (Davis *et al.*, 2001; Hatzivassiliou *et al.*, 2001; Miller *et al.*, 2002) and the presence of one or more functional immunoreceptor tyrosine-based activation (ITAM) or inhibitory (ITIM) signalling motifs (Ehrhardt *et al.*, 2003; Leu *et al.*, 2005), is consistent with an immunoregulatory role. A link between polymorphisms in *FCRL*s and autoimmune disease is also suggestive of such a function (Ikari *et al.*, 2006; Kochi *et al.*, 2005; Umemura *et al.*, 2006).

The putative extracellular Ig domains of chFcR/L have up to 48% amino acid identity with Ig domains of the mammalian FcR and FCRL, which is consistent with the level of conservation of other immune-related homologues observed between birds and mammals (Hillier *et al.*, 2004; Kaiser *et al.*, 2005).

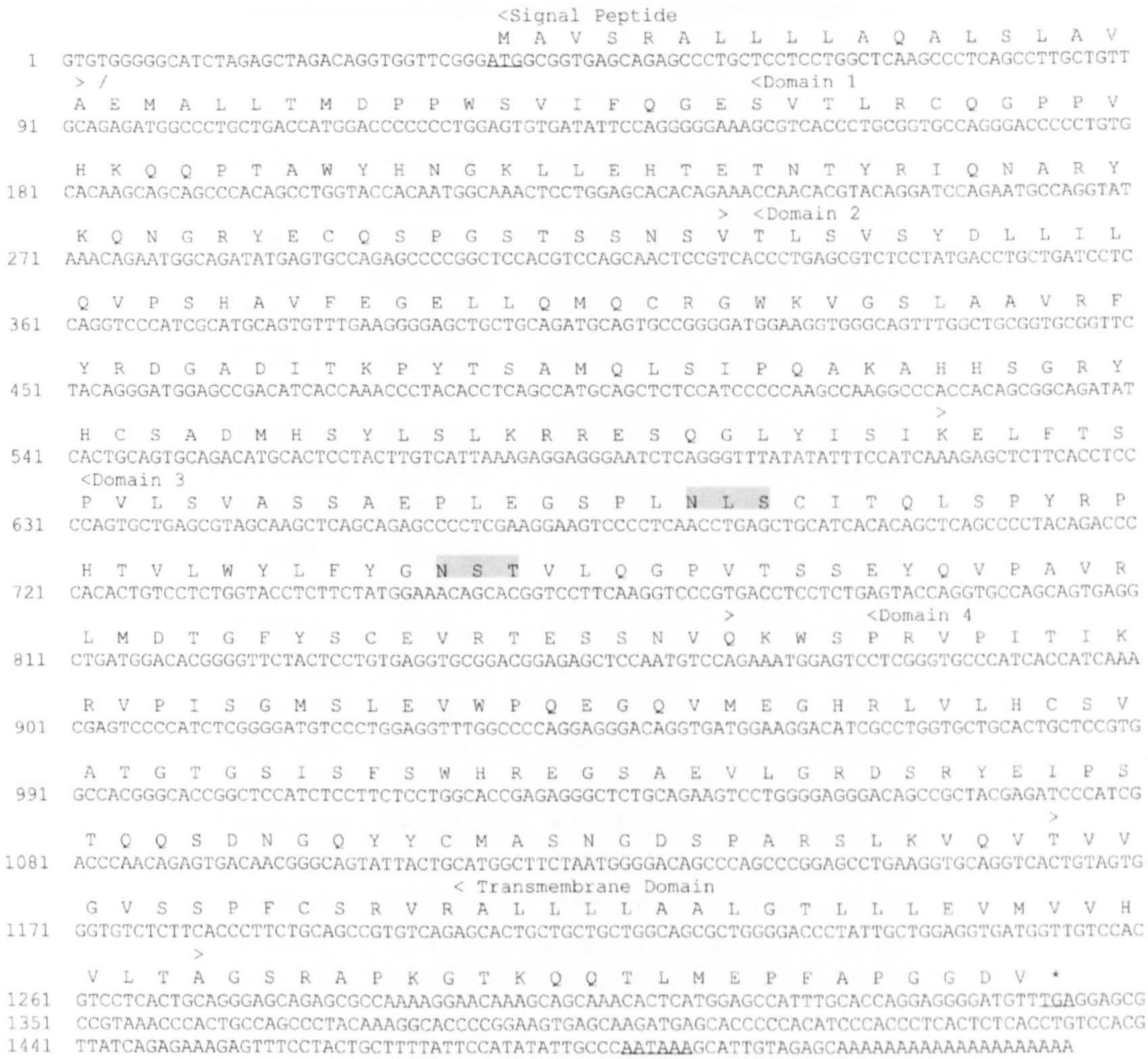


Figure 6.3.3 i
The complete coding sequence (CDS) of chFcR/L

Nucleotide sequence (numbered) and predicted amino acid sequence (line above) of cloned chFcR/L transcript cDNA. Predicted signal peptide, immunoglobulin domains and transmembrane region are marked (<> above amino acid sequence). Predicted signal peptide cleavage site is marked (/). Predicted N-linked glycosylation sites are highlighted in grey. Initiation and termination codons and polyadenylation sequence are underlined.

Receptor		Accession N°	Score	E Value
FCRL4	Human	AAK93970	212	2e-53
FCRL3	Human	CAH73059	212	2e-53
FcgRI	Mouse	BAD97676	174	7e-42
FcgRI	Dog	NP_001002976	174	9e-42
FcgRI	Macaque	AAL92095	174	1e-41
MMAN-g	Mouse	BAB18569	173	1e-41
FCRL2	Human	CAH73062	172	3e-41
FCRLS	Mouse	NP_109632	171	4e-41
FcgRI	Human	NP_000557	162	2e-38
MGC82906	Xenopus	AAH73602	160	9e-38
FCRL5	Mouse	NP_899045	158	5e-37
FcgRI	Bovine	AAI12692	155	3e-36
XFL1.6	Xenopus	AAQ63873	153	2e-35
FCRL5	Human	AAI01070	139	4e-31
MGC82866	Xenopus	AAH78060	135	5e-30
XFL1.4	Xenopus	AAQ56585	135	5e-30
XFL1.2b	Xenopus	AAQ56585	133	2e-29
FCRLB	Mouse	NP_001025155	127	1e-27
Fcgr1-prov	Xenopus	AAH88875	126	2e-27
FCRLA	Human	CAH74158	123	2e-26
FCRL1	Human	AAH33690	119	2e-25
FCRL6	Human	NP_001004310	118	5e-25
XFL1.5	Xenopus	AAQ56586	109	2e-22
FceRI	Human	1F6A_A	100	1e-19
XFL1.1b	Xenopus	AAQ56584	99.0	4e-19
XFL1.2a	Xenopus	AAQ56582	97.8	9e-19
XFL1.3	Xenopus	AAQ63874	96.3	2e-18
FcgRII	Guinea Pig	A34636	92.4	4e-17
FcgRII	Human	AAH20823	91.7	6e-17
FCRLB	Human	AAT77991	91.7	5e-17
FcgRII	Macaque	AAL92097	90.1	2e-16
XFL2	Xenopus	AAQ56587	88.6	6e-16
FcgRIII	Bovine	CAA68026	87.4	1e-15
FcgRII	Mouse	AAH38070	85.5	5e-15
FcgRIII	Mouse	NP_034318	84	1e-14
FcgRIV	Mouse	NP_653142	83.2	2e-14
FceRI	Mouse	NP_034314	82.8	3e-14
FcRI	Catfish	ABB89215	81.6	6e-14

Table 6.3.3 ii
Homologues of chFcR/L

The top hits from a BLAST search of the NCBI non-redundant protein database show that chFcR/L is closely related to Fc receptors (Fc_R) and Fc receptor-like (FCRL) sequences from mammals, amphibians and bony fish.

6.3.4 Analysis of *chFcR/L* exon structure

The complete cDNA sequence of chFcR/L was used to re-screen the chicken genome. Two contigs were identified in addition to the two described in section 6.3.1, which extended the genomic coverage and allowed the gene structure of *chFcR/L* to be deduced. The contigs were unassigned to any chromosome in the February 2004 release of the chicken genome, but have since been anchored to chromosome 25 in the March 2006 assembly. However, in both assemblies they are incorrectly ordered. The correct order was determined by alignment of overlapping sequences (Figure 6.3.4).

At ~3670bp, *chFcR/L* is smaller than any mammalian FcR or FCRL gene due to introns that, whilst they conform to the GT-AG rule (Breathnach *et al.*, 1978; Catterall *et al.*, 1978), are less than 25% of the size of their mammalian counterparts. *chFcR/L* has 7 exons, with exons III, IV, V and VI encoding one extracellular Ig domain each (EC_{1-4}); the exons follow the phase 1 splicing typical for Ig & Ig-like domains (Davis *et al.*, 2002; Ravetch and Kinet, 1991). The transmembrane domain (TM), cytoplasmic tail (C) and 3' untranslated region (UT) are all encoded by exon VII. The signal peptide is split between two exons: S_1, which also encodes the 5'UT and the ATG translation initiation codon, and S_2. S_2 is 21bp in length, a feature that distinguishes members of the FcR and FCRL families from other Ig-containing immunoreceptor gene families, such as the LRC (Davis *et al.*, 2002).

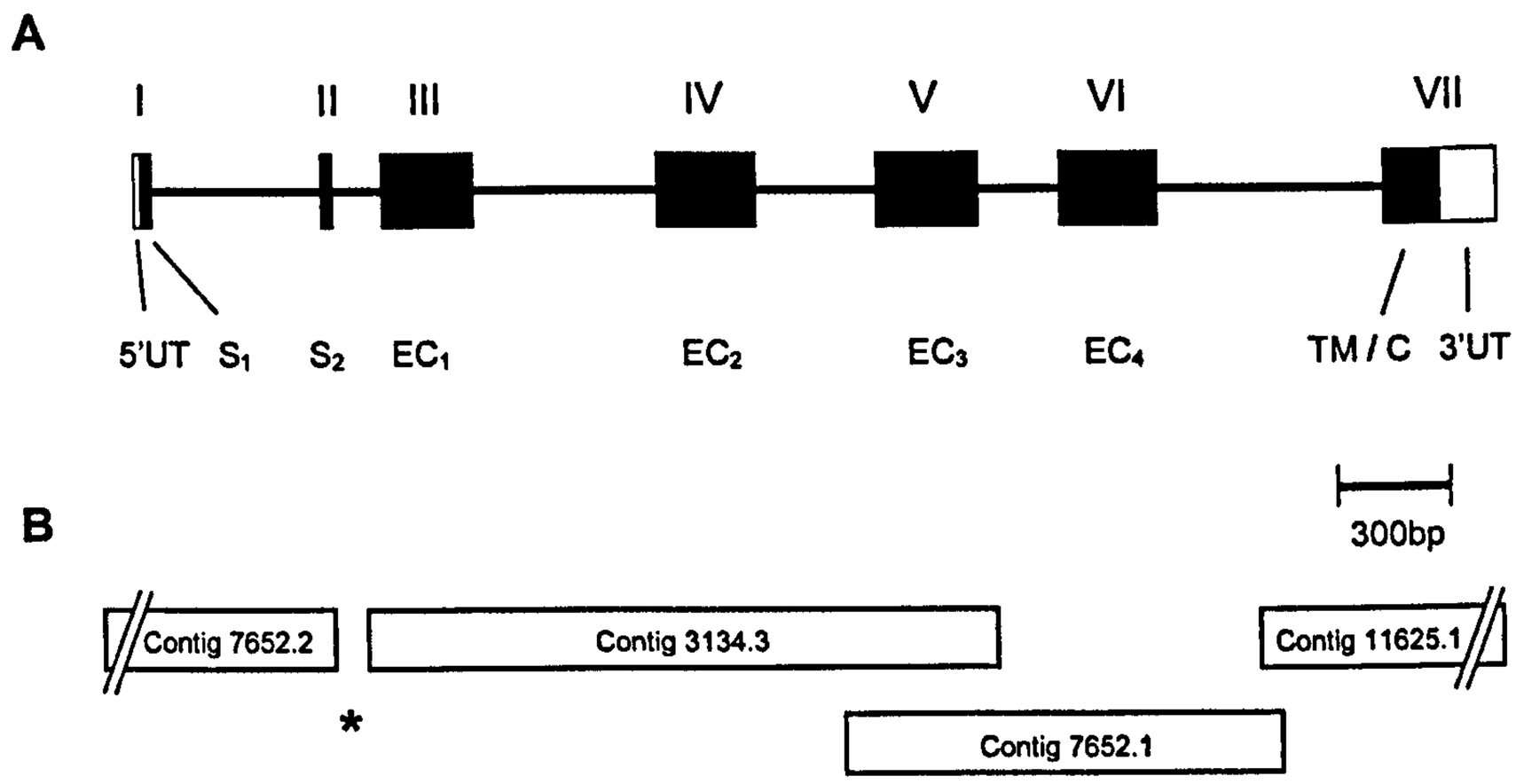


Figure 6.3.4
Exon structure of *chFcR/L* and coverage by the published chicken genome

(A) Introns are shown as lines, exons as boxes (filled denotes putative translated sequence). UT = Untranslated region, S = Exon encoding part of signal peptide, EC = Exon encoding extracellular immunoglobulin domain, TM = Exon encoding transmembrane domain, C = Exon encoding cytoplasmic region. (B) Corrected order of contig coverage of chFcR/L in the chicken genome. 2004 assembly ID codes are shown. (//) denotes continuation of contig. *The gap between contigs 7652.2 and 3134.3 was covered by an immature transcript, which had not yet had any introns spliced out (the ~3.5kb band in figure 6.3.5 A lane 1).

6.3.5 Splice variants of chFcR/L

cDNA was prepared by reverse transcription of total RNA isolated from MQ-NCSU chicken monocytes and crude preparations of chicken basophils. Primers complementary to 5' and 3' sequences of the chFcR/L CDS were then used to PCR amplify transcript variants differing in which splice sites had been joined (Figure 6.3.5).

Although several chFcR/L transcripts containing introns were isolated from both MQ-NCSU cells and the basophil preparation (unmarked bands in Figure 6.3.5 A), three distinct splice variants were identified with a marked difference in their

distribution (Figure 6.3.5): The 'complete' chFcR/L transcript and a variant lacking the second Ig domain, chFcR/L ΔEC_2, were cloned from MQ-NCSU monocytes but were not detected in the basophil preparation. Conversely, a variant lacking 67 nucleotides from the same domain, which causes a reading frame shift leading to an early termination (chFcR/L $\Delta EC_{2'/3/4}$), was isolated from the basophil preparation but was not detected in MQ-NCSU cells. As this sequence lacks the transmembrane and cytoplasmic domains, the translated protein would be secreted, rather like a splice variant of a low affinity IgG receptor (Teillaud *et al.*, 1994). Despite the possibility that this sequence was derived from a different cell type due to contamination of the preparation (predominantly with red blood cells), its existence nevertheless suggests a physiological role. Others researchers have subsequently detected all three chFcR/L variants, but no others (Fayngerts *et al.*, 2007).

The presence of three different forms of chFcR/L could represent a higher level of sophistication in ligand interaction and regulation. It should be noted, however, that although numerous transcript variants have been described for the FcγRs, few are both translated and expressed on the cell surface (Ernst *et al.*, 1998; van Vugt *et al.*, 1999).

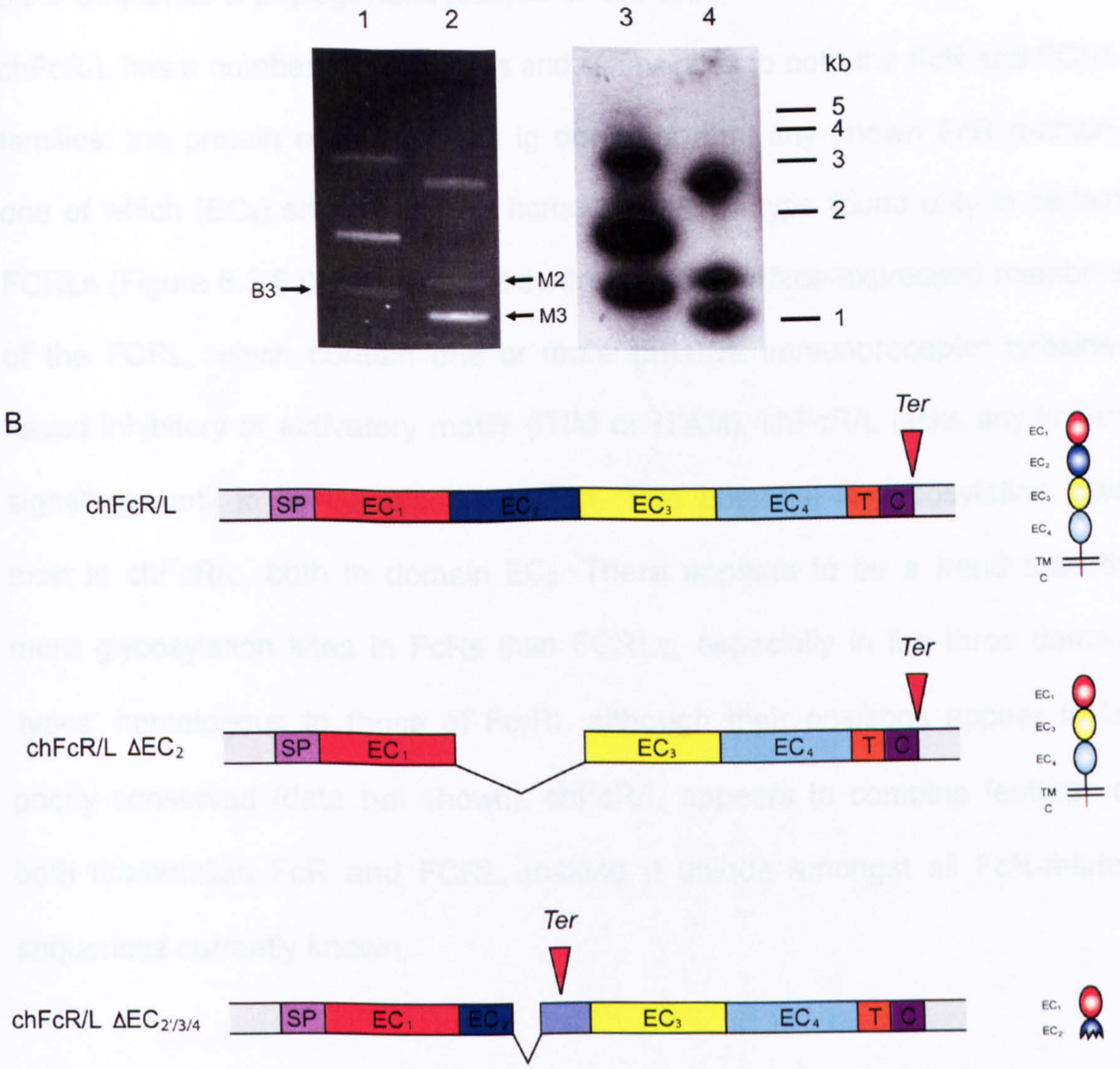


Figure 6.3.5
Identification of chFcR/L transcript splice variants

(A) 1% agarose showing products of PCR reactions using chFcR/L-specific primers stained with ethidium bromide / UV transillumination (left, 1 & 2) or Southern blotted with radiolabelled probes complementary to EC_3 (right, 3 & 4). Lanes 1 & 3, products of PCR reaction using cDNA derived from chicken blood basophil RNA. Lanes 2 & 4, products of PCR reaction using cDNA derived from MQ-NCSU chicken monocytes. The labelled bands were identified as mature transcript splice variants by gel extraction and cloning using a TOPO TA vector, pCR4 (Invitrogen). M2, M3 and B3 sequences were found to be complete transcript, ΔEC_2 and $\Delta EC_{2'/3/4}$ respectively. Unmarked bands were immature transcripts containing unspliced introns. Predicted features of each sequence are shown (B) with cartoons depicting the encoded protein. SP = Signal peptide, EC = Extracellular immunoglobulin domain, T = Transmembrane domain, C = Cytoplasmic region, Ter = Termination codon.

6.3.6 Sequence & phylogenetic analysis of chFcR/L

chFcR/L has a number of similarities and differences to both the FcR and FCRL families: the protein contains more Ig domains than any known FcR α-chain, one of which (EC_4) shares highest homology with a type found only in certain FCRLs (Figure 6.3.6 i). However, in contrast to all surface-expressed members of the FCRL, which contain one or more putative immunoreceptor tyrosine-based inhibitory or activatory motifs (ITIM or ITAM), chFcR/L lacks any known signalling motif in its cytoplasmic region. Two potential N-glycosylation sites exist in chFcR/L, both in domain EC_3. There appears to be a trend towards more glycosylation sites in FcRs than FCRLs, especially in the three domain 'types' homologous to those of FcγRI, although their positions appear to be poorly conserved (data not shown). chFcR/L appears to combine features of both mammalian FcR and FCRL, making it unique amongst all FcR-related sequences currently known.

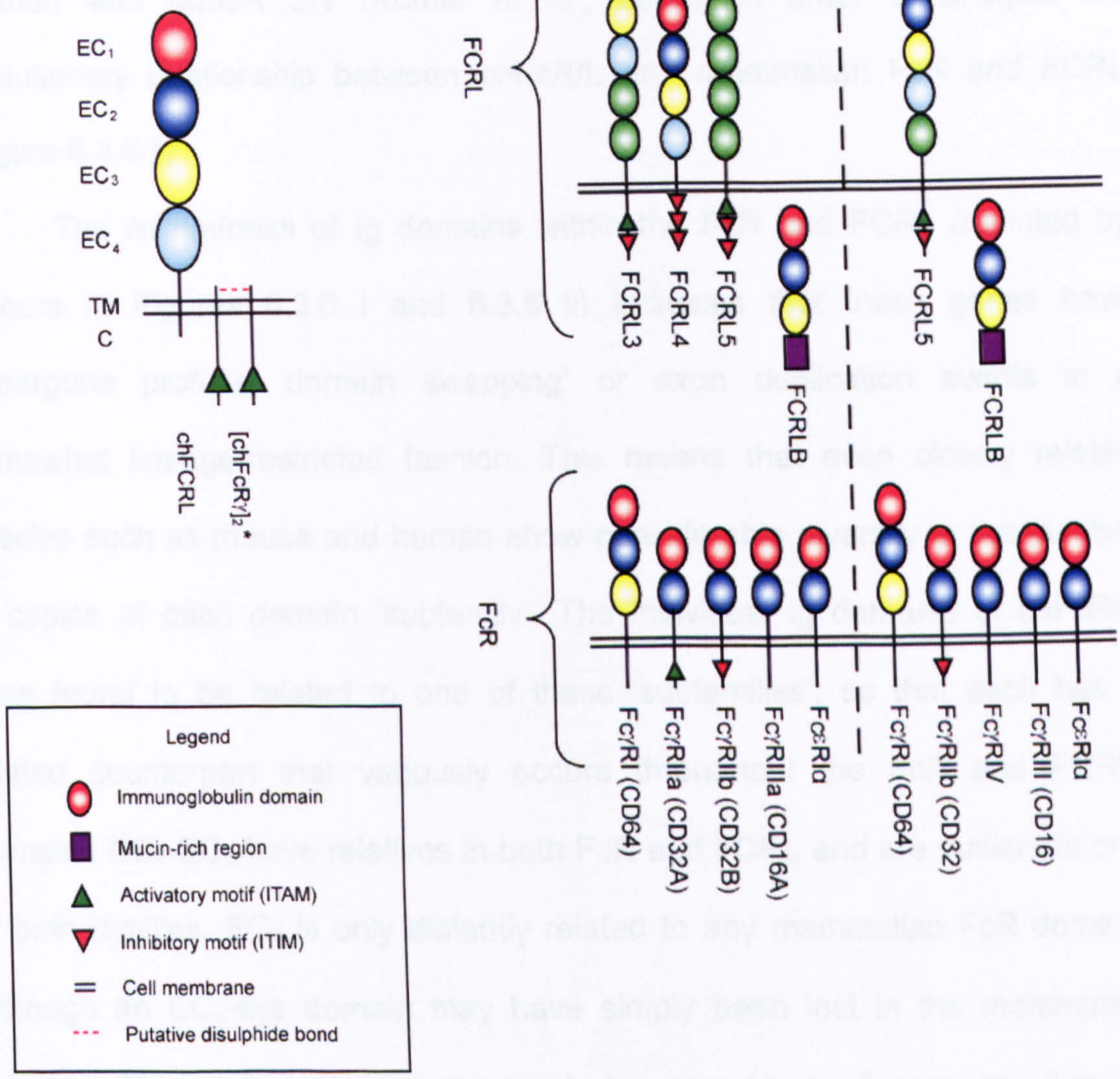


Figure 6.3.6 i
chFcR/L contains Ig domains found in mammalian FcR and FCRL

Left, schematic representation of chFcR/L in the cell membrane (left), putatively in association with a signalling subunit, the chicken orthologue of FcRγ, which is shown as a disulfide-linked homodimer as occurs in mammalian FcR complexes. EC = extracellular Ig domain, TM = transmembrane domain, C = cytoplasmic region.
Right, schematic representations of mammalian FcR and selected FCRL with the longest stretches of alignment to chFcR/L. Ig domains are coloured according to their relatedness to chFcR/L.

Amino acid sequences of individual Ig domains of chFcR/L, mammalian FcR, FCRL, catfish IpFcRI and an outlier only distantly related to the FcR family, CD89, were aligned using ClustalW (Thompson *et al.*, 1994). A phylogenetic tree was drawn using the neighbour-joining bootstrap resampling method with MEGA 3.1 (Kumar *et al.*, 2004), in order to analyse the evolutionary relationship between chFcR/L and mammalian FcR and FCRL (Figure 6.3.6 ii).

The relatedness of Ig domains within the FcR and FCRL (denoted by colours in Figures 6.3.6 i and 6.3.6 ii) indicates that these genes have undergone profuse 'domain swapping' or exon duplication events in a somewhat lineage-restricted fashion. This means that even closely related species such as mouse and human show considerable diversity in the number of copies of each domain 'subfamily'. The individual Ig domains of chFcR/L were found to be related to one of these 'subfamilies', so that each has a related counterpart that variously occurs throughout the FcR and FCRL. Domains EC_1-EC_3 have relatives in both FcR and FCRL and are outliers to one or both families. EC_4 is only distantly related to any mammalian FcR domain, although an EC_4-like domain may have simply been lost in the mammalian lineage. These factors, compounded by sequence divergence due to evolutionary distance, means that the analysis cannot determine absolutely whether *chFcR/L* is more closely related to one family than the other, although a common ancestry seems certain.

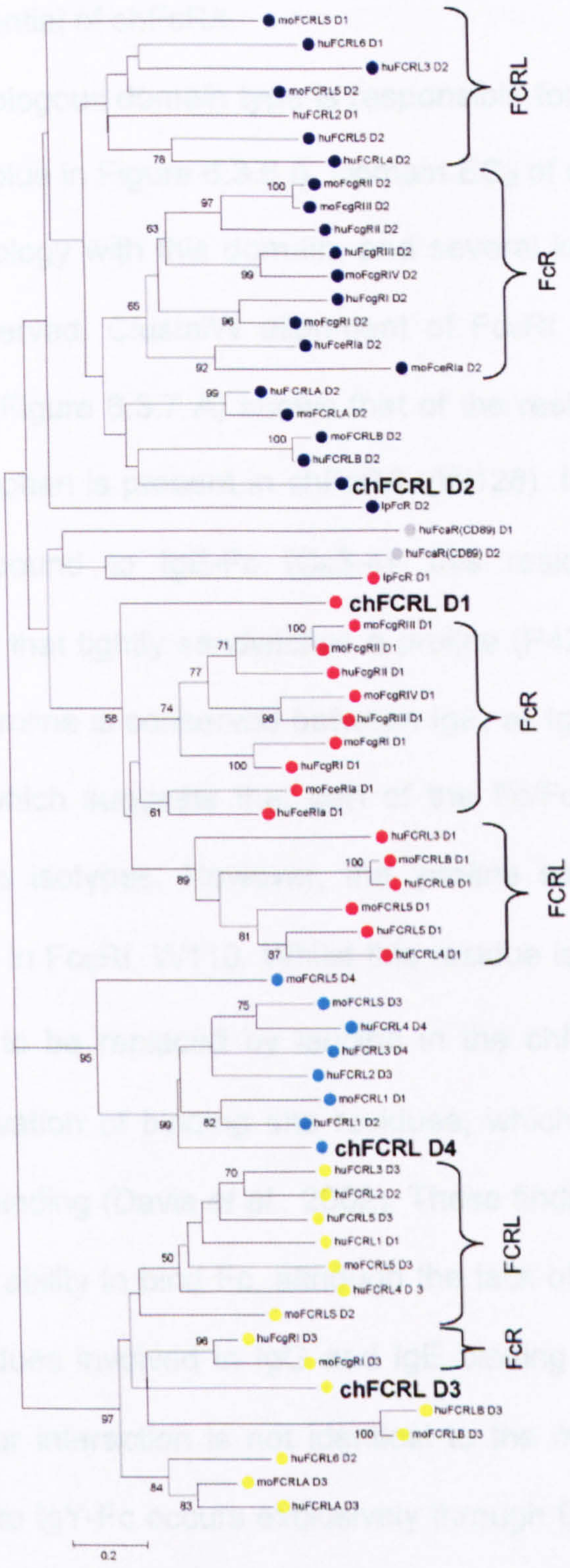


Figure 6.3.6 ii
Evolutionary relationships of Ig domains in chFcR/L, Fc receptors and FCRL

Phylogenetic tree of individual Ig domains from chFcR/L, catfish IpFcRI and all currently identified mammalian FcR & FCRL. The tree was drawn using the neighbour joining method with 1000 bootstrap replicates. Domains are coloured according to their relatedness.

6.3.7 Fc binding potential of chFcR/L

A single homologous domain type is responsible for Ig binding in each of the FcRs (coloured blue in Figure 6.3.6 i). Domain EC_2 of chFcR/L was found to share greatest homology with this domain, and several key residues (Davis *et al.*, 2002) are conserved. ClustalW alignment of FcεRI and FcγRI-III binding sites with chFcR/L (Figure 6.3.7 A) shows that of the residues implicated in Fc contact, only a trytophan is present in chFcR/L (W128). In the crystal structure of human FcεRI bound to IgE-Fc (Cε3-4), this residue forms part of a hydrophobic pocket that tightly sandwiches a proline (P426) in the Fc (Garman *et al.*, 2000). This proline is conserved between IgE, all IgG subclasses and IgY (Figure 6.3.7 B), which suggests that part of the Fc/FcR interaction may be similar for all three isotypes. However, the 'proline sandwich' also involves another tryptophan in FcεRI, W110. Whilst this residue is well conserved in the FcγRs, it appears to be replaced by leucine in the chFcR/L sequence. Most FCRL lack conservation of binding site residues, which helps to explain their alleged lack of Ig binding (Davis *et al.*, 2002). These findings could suggest that chFcR/L lacks the ability to bind Fc, although the lack of conservation in IgY of several other residues involved in IgG and IgE binding strongly suggests that the IgY/Fc receptor interaction is not identical to the mammalian equivalents. Indeed, if binding to IgY-Fc occurs exclusively through EC_2 in the same way as mammalian FcRs, the presence of splice variants lacking most, if not all of this domain would be somewhat inexplicable (see section 6.3.5). EC_1 is the only domain to occur in all three chFcR/L variants, so it might be argued that this is a more reasonable candidate for the Fc binding domain. However, a splice variant of a receptor for IgA, FcαR has been observed lacking the binding domain,

which also happens to be the second extracellular domain (Suzuki *et al.*, 1999). Regulation of ligand binding by transcriptional control of receptor was postulated to explain the appearance of these variants, and this may true of chFcR/L also.

A

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chFcR/L  : YDLLILQVPSHAVFEGELLQMQCRGWKVGSLAAVRFYRDGADITKPYTSAMQLSIPQAKA 162
FcgRI    : RGWLLLQVSSRVFTEGEPLALRCHAWKDKLVYNVLYYRNGKAFKFF-HWNSNLTILKTNI 160
FcgRII   : SEWLVLQTPHLEFQEGETIVLRCHSWKDKPLVKVTFFQNGKSKKFS-RSDPNFSIPQANH 178
FcgRIII  : IGWLLLQAPRWVFKEEDPIHLRCHSWKNTALHKVTYLQNGKGRKYF-HHNSDFYIPKATL 200
FceRIa   : SDWLLLQASAEVVMEGQPLFLRCHGWRNWDVYKVIYYKDGEALKYW-YENHNISITNATV 143

chFcR/L  : HHSGRYHCSADMHSYLSLK-RRESQGLYISIK-                             193
FcgRI    : SHNGTYHCSG-MGKHR-----YTSAGISVTVK-                             186
FcgRII   : SHSGDYHCTGNIGYTL-----YSSKPVTITVQ-                             205
FcgRIII  : KDSGSYFCRGLFGSKN-----VSSETVNITITQ                             228
FceRIa   : EDSGTYYCTGKVWQLD-----YESEPLNITVIK                             171
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B

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Gamma1   : ----------------------------------------LLGGPSVFLFPPKPKDTLM 252
Epsilon  : SELTLSQKHWLSDRTYTCQVTYQG--HTFEDSTKKCAD--SNPRGVSAYLSRPSPFD-LF 349
Upsilon  : SRVNVSGTDWREGKSYSCRVRHPATNTVVEDHVKGCPDGAQSCSPIQLYAIPPSPGE-LY 363

Gamma1   : ISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD 312
Epsilon  : IRKSPTITCLVVDLAPSKGTVNLTWSRASGKPVNHSTRKEEKQRNGTLTVTSTLPVGTRD 409
Upsilon  : ISLDAKLRCLVVNLP-SDSSLSVTWTREKSGNLRPDPMVLQEHFNGTYSASSAVPVSTQD 422

Gamma1   : WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGF 372
Epsilon  : WIEGETYQCRVTHPHLPRALMRSTTKTSGPRAAPEVYAFATPEWPGSRDKRTLACLIQNF 469
Upsilon  : WLSGERFTCTVQHEELPLPLSKSVYRNTGPTTPPLIYPFAPHPEELSLSRVTLSCLVRGF 482
```

Figure 6.3.7
Alignment of binding sites in IgG and IgE Fc regions and their receptors with homologous sequences in IgY and chFcR/L

Amino acid sequences aligned with ClustalW. (A) chFcR/L (EC_2) and binding sites in Fc receptors whose ligand interactions are supported by crystallographic data. (B) IgY (upsilon) heavy chain (Cυ3) and binding sites in IgG1 (gamma1) and IgE (epsilon) heavy chains. Residues making direct contact with Fc or receptor are highlighted. Conserved contact residues are highlighted in red.

6.4 Identification and cloning of a putative chicken FcRγ

In mammals, the FcRγ subunit was initially identified as part of the FcεRI complex (Blank *et al.*, 1989; Perez-Montfort *et al.*, 1983), but has subsequently been found to be a common component of most Fc receptor complexes and provides their means of intracellular signalling (Hulett and Hogarth, 1994). The α-chains of FcεRI and FcγRIII have been found to require interaction with FcRγ in order to be expressed on the cell surface (Kurosaki and Ravetch, 1989; Miller *et al.*, 1989; Ra *et al.*, 1989). FcγRIα can be expressed transiently in the absence of FcRγ (Allen and Seed, 1989), although they are associated *in vivo* and may be required for maintenance of expression and proper function (Ernst *et al.*, 1993; Masuda and Roos, 1993; Miller *et al.*, 1996; Takai *et al.*, 1994; van Vugt *et al.*, 1996).

Orthologues of mammalian FcRγ have been identified in a teleost (*Cyprius carpio)* and an amphibian (*Xenopus laevis*), indicating that this subunit appears to be well conserved throughout the jawed vertebrates (Guselnikov *et al.*, 2003). At the time of these experiments, there was no record of an avian FcRγ orthologue in the literature, but these findings and the presence of a closely related signalling subunit, TCRζ, in the chicken (Gobel and Bolliger, 1998), suggested that one may exist.

In order to be able to test the possibility of subunit dependence for *in vitro* expression of chFcR/L and to expand the range of possible future experiments should this be the case, an orthologue of FcRγ was identified and cloned. Independent investigators have subsequently reported cloning of the same sequence (Viertlboeck *et al.*, 2005).

6.4.1 Data mining of chicken genome and EST libraries

In a similar approach to the identification of chFcR/L, the amino acid sequence of mammalian FcRγ was used to BLAST search the chicken genome and EST libraries. Whilst the genome did not contain any candidate sequences, several ESTs were identified using NCBI dbEST (Boguski *et al.*, 1993). Translated amino acid sequences from these ESTs gave a best reciprocal hit to mammalian FcRγ sequences, indicating that the encoded protein is very likely to be the orthologue of FcRγ in the chicken. Consistent with this assertion, other returned BLAST hits included the adaptor subunit CD3/TCRζ, (Gobel and Bolliger, 1998), which is closely related to FcRγ.

6.4.2 Cloning of putative chFcRγ

Primers were designed from the identified ESTs and the complete coding sequence amplified by PCR from MQ-NCSU-derived cDNA (Figure 6.4.2 A) and cloned into a TOPO TA vector, pCR4 (Invitrogen). The translated amino acid sequence of chFcRγ was aligned to those of FcRγ orthologues from several mammals as well as frog (Guselnikov, 2004) and carp (Fujiki *et al.*, 2000) using ClustalW (Figure 6.4.2 B).

The chicken orthologue shows surprisingly high sequence identity to human (62%), which is even higher in the transmembrane region (85%) and the ITAM motif (72%). This is likely to be reflection of this molecule's crucial role, providing the capacity to initiate signalling cascades in numerous immune receptor complexes. The interaction between ITAM motifs and tyrosine kinase SH2 domains does not tolerate mutations well, and so has therefore been

highly conserved between vertebrate classes. Interestingly, absolute conservation of all residues implicated in key protein: protein interactions in the transmembrane domain (Figure 6.4.2 B) implies that the chicken FcRγ would form a homodimer (as shown in figure 6.3.6 i) and make contacts with other receptor subunits in an identical fashion to its mammalian orthologues. Although there does not appear to be a common motif required for FcRγ interaction, the α-chains of FcεRI, FcγRI and FcγRIII contain a charged residue in their transmembrane regions. FcγRIIα, which does not interact with FcRγ, has no such residue. The chFcR/L transmembrane region contains glutamic acid (-), arginine (+) and histidine (+), which were predicted by SOSUI (Hirokawa *et al.*, 1998) to be on opposite faces of the transmembrane helix, and thus gamma subunit interaction appeared to be possible.

A

```
                                                          <Signal Peptide
                                                          M  S  A  W  R  L  W  A  A
  1 ACGCGGGGAGGGGAAGGCTCTCAGCCTCAGCTCTGCCTCCTCCTCCACGGCTCCGTGACACCATGAGCGCCTGGCGGCTGTGGGCCGCCG
                                        > /         <Transmembrane Domain
    A  L  L  L  Q  V  S  A  A  E  A  L  A  E  P  E  L  C  Y  V  L  D  G  I  L  F  L  Y  G  I
 91 CTCTGCTGCTCCAGGTGTCGGCAGCGGAGGCACTGGCAGAGCCGGAGCTGTGCTACGTCCTTGATGGGATCCTCTTCCTCTACGGCATCG
                        >
    V  L  T  I  L  Y  C  R  L  K  F  M  T  R  R  A  L  Q  E  K  A  K  K  P  E  E  G  I  Y  T
181 TCCTCACCATCCTCTACTGTCGCCTCAAGTTCATGACCCGCAGAGCACTGCAGGAGAAGGCCAAGAAGCCAGAAGAAGGCATCTACACAG

    G  L  S  T  E  H  Q  E  T  Y  E  M  L  Q  H  K  A  *
271 GGCTCAGCACTGAGCACCAGGAGACGTACGAAATGCTGCAGCACAAAGCCTGAGTTCGGACCCCCCCACCACCACCACCGCTGCGCCCCC
361 TCCCCAAATTACTGCTTTTGGGGCTACGATGCAGAATTGGAGCTTTTCCCACCCAATAAATGGAGGTTTTCAGCCTCAAAAAAAAAAAAA
451 AAAAAAAAAAAAAA
```

B

```
                    <      TM region      >
Chicken  : EPELCYVLDGILFLYGIVLTILYCRLKFMTRRALQEK------------
Human    : EPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITS------------
Boar     : EPQLCYILDAILFLYGIVLTLLYCRLKLQVRKAAIDS------------
Dog      : EPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAVTS------------
Mouse    : EPQLCYILDAVLFLYGIVLTLLYCRLKIQVRKAAIAS------------
Opposum  : EPQLCYILDAVLFIYGIVLTLLYCRLKIQMRKAAAR------------
Frog     : EPEICYILDAILFLYGIVLTALYCHLKIQTKKAQKSKPAGAIYEHLNYPE
Carp     : DGGVCYILDGILIVYGIVLTILYCRLKMRSSGDKAFS------------

                                <      ITAM      >
Chicken  : ---AKKP--------EEGIYTGLSTEHQETYEMLQ-HKA----
Human    : ---YEKS---------DGVYTGLSTRNQETYETLK-HEKPPQ-
Boar     : ---YEKS---------DGVYTGLSTRNQETYETLK-HEKPPQ-
Dog      : ---YEKP---------DGIYTGLSTRNQETYETLK-HEKPPQ-
Mouse    : ---REKA---------DAVYTGLNTRSQETYETLK-HEKPPQ-
Opposum  : ---YEKS---------EEVYTGLSTRTQETYETLK-YEKSPQ-
Frog     : KQIYEKIGEEAQKKGEEGEYTGLESVDKGTYETIK-GESKKN-
Carp     : ----EKR--------EGDIYQDLGRRDVDTYDTLHPGKKKPLA
```

Figure 6.4.2
A putative orthologue of the FcRγ subunit in the chicken

(A) Nucleotide sequence (numbered) and predicted amino acid sequence (line above) of cloned chFcRγ transcript cDNA. Predicted signal peptide and transmembrane region are marked (<> above amino acid sequence). Predicted signal peptide cleavage site is marked (/). A canonical ITAM motif is highlighted in grey. Initiation and termination codons and polyadenylation sequence are underlined.
(B) ClustalW alignment of amino acid sequences of various FcRγ orthologues. Residues in the transmembrane (TM) region of human FcRγ have been studied by mutagenesis (Wines *et al.*, 2006): those responsible for subunit homodimerisation are highlighted in black, those required for FcεRIα association are highlighted in green and those which interact with FcαRI are shown in red. Tyrosines involved in canonical ITAM motifs are highlighted in blue. Frog FcRγ has two putative ITAM motifs (Guselnikov *et al.*, 2003).

6.5 Expression of chFcR/L and chFcRγ

6.5.1 Adaptation of sequences for expression with N-terminal epitope tags in mammalian cells

The full length chFcR/L and chFcRγ cDNA sequences (previously cloned in pCR4-TOPO) were used as templates in PCR reactions with primers designed to incorporate restriction sites 5' and 3' of the coding sequence for the mature peptide. The chFcR/L and chFcRγ sequences were amplified with the addition of sequences encoding in-frame N-terminal V5 and FLAG epitope tags between the codons for the predicted signal peptide cleavage site (Figure 6.5.1 A & B). Epitope tags are short amino acid motifs for which specific antibodies are commercially available and which have the advantage over larger tags (such as Green Fluorescent Protein) that they are less likely to cause structural or functional artefacts. Both of these tags have been successfully used in similar applications: an N-terminal V5-tagged receptor containing Ig domains (Viertlboeck *et al.*, 2005) and an N-terminal FLAG-tagged gamma subunit (Wu *et al.*, 2000).

The sequences were cloned into the pRY mammalian expression vector (Young *et al.*, 1995), following the same cloning strategy used for the IgY-Fc cDNA sequences (see chapter 4, figure 4.3.1 iii); the B72.3 Vκ light chain leader sequence and epsilon heavy chain untranslated region were ligated 5' and 3' of the chFcR/L CDS respectively (Figure 6.5.1 C).

A

1. Natural leader sequence / chFcR/L N-terminus:

5'
MET...ALA VAL ALA / GLU MET ALA LEU LEU...
ATG...GCT GTT GCA / GAG ATG GCC CTG CTG...

2. B72.3 leader sequence-adapter / V5-tag - chFcR/L N-terminus:

5' ↓ V5
MET...CYS ASP ILE / GLY LYS PRO ILE PRO ASN PRO
ATG...TGT **GAT ATC** / GGT AAG CCT ATC CCT AAC CCT
EcoRV

LEU LEU GLY LEU ASP SER THR-GLU MET ALA LEU LEU...
CTC CTC GGT CTC GAT TCT ACG-GAG ATG GCC CTG CTG...

3. Natural chFcR/L C-terminus / stop-UTR:

5'
...GLY ASP VAL *Ter*
...GGG GAT GTT TGA GGA GCG CCG TAA ACC CAC TGC...

4. chFcR/L C-terminus/stop-adapter-B72.3 UTR:

5'
...GLY ASP VAL *Ter*
...GGG GAT GTT TGA **GTT TAA ACC** TGC CTC CCT...
PmeI

B

1. Natural leader sequence / chFcRγ N-terminus:

5'
MET...ALA LEU ALA / GLU PRO ALA LEU CYS...
ATG...GCG GAG GCA / GAG CCG GCC CTG TGC...

2. B72.3 leader sequence-adapter / FLAG-tag - chFcRγ N-terminus:

5' ↓ FLAG
MET...CYS ASP ILE / ASP TYR LYS ASP ASP ASP ASP
ATG...TGT **GAT ATC** / GAC TAT AAG GAC GAT GAT GAC
EcoRV

LYS-GLU PRO GLU CYS...
AAG-GAG CCG GAG TGC...

3. Natural chFcRγ C-terminus / stop-UTR:

5'
...HIS LYS ALA *Ter*
...CAC AAA GCC TGA GTT CGG ACC CCC CCA CCA CCA...

4. chFcRγ C-terminus/stop-adapter-B72.3 UTR:

5'
...HIS LYS ALA *Ter*
...CAC AAA GCC TGA **GTT TAA AC**C TGC CTC CCT...
PmeI

C

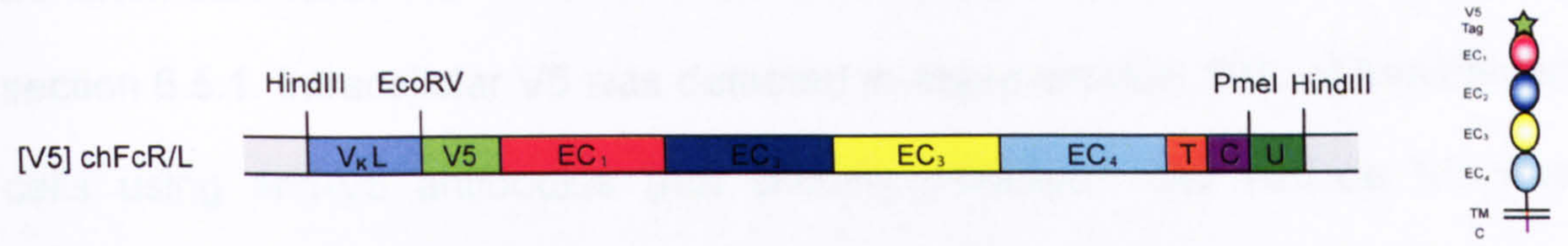


Figure 6.5.1 (above and previous two pages)
Adaptation of chFcR/L and chFcRγ cDNA sequences for mammalian expression with N-terminal epitope tags

Nucleotide and encoded amino acid sequences of regions modified by PCR in chFcR/L (A) and chFcRγ (B). Bases around the original predicted signal peptide cleavage site (1) were altered to include an N-terminal V5 or FLAG tag and an EcoRV restriction site (shown bold & underlined), which was used to ligate the mature peptide coding sequence 3' of the mouse B72.3 Vκ light chain leader sequence (2). Similarly, bases around the original termination codon (3) were altered to include a PmeI restriction site, which was used to ligate the mature peptide coding sequence to the 5' end of the epsilon heavy chain untranslated region. (C) A summary schematic showing the encoded elements of the generated expression cassettes, with representations of the expected proteins. VκL = leader sequence (encoding signal peptide), V5/FLAG = epitope tag, EC = extracellular Ig domain, T/TM = transmembrane region, C = cytoplasmic region, U = untranslated region.

6.5.2 Transient expression of chFcR/L

The feasibility of the [V5-chFcR/L] expression cassette was tested by transient transfection of CHO-K1 cells with the pRY-based vector prepared in section 6.5.1. Intracellular V5 was detected in approximately 30% of transfected cells using anti-V5 antibodies (not shown). However, cell surface V5 was detected when cells were co-transfected with the [V5-chFcR/L] and [FLAG-chFcRγ] pRY vectors, but not when cells were transfected with [V5-chFcR/L] vector alone (Figure 6.5.2).

This finding not only appears to confirm that the tagged constructs are capable of expression, but also that insertion of chFcR/L in the cell membrane requires an accessory subunit. Association with an ITAM-containing gamma subunit would explain how chFcR/L acquires signalling potential *in vivo*. As outlined above, the high affinity receptors for IgG (FcγRIα) and IgE (FcεRIα) also associate with FcRγ, although unlike FcεRIα and chFcR/L, FcγRIα can be expressed (albeit transiently) in its absence.

Presumably due to the difficulty of achieving co-expression of both chFcR/L and chFcRγ in the same cell, less than 5% of cells were positive for chFcR/L surface expression. IgY binding potential was tested, but due to the low level of surface expression and a fairly high level of background in this assay format (as judged by IgY/anti-IgY-FITC staining of untransfected CHO-K1 cells), the results were inconclusive.

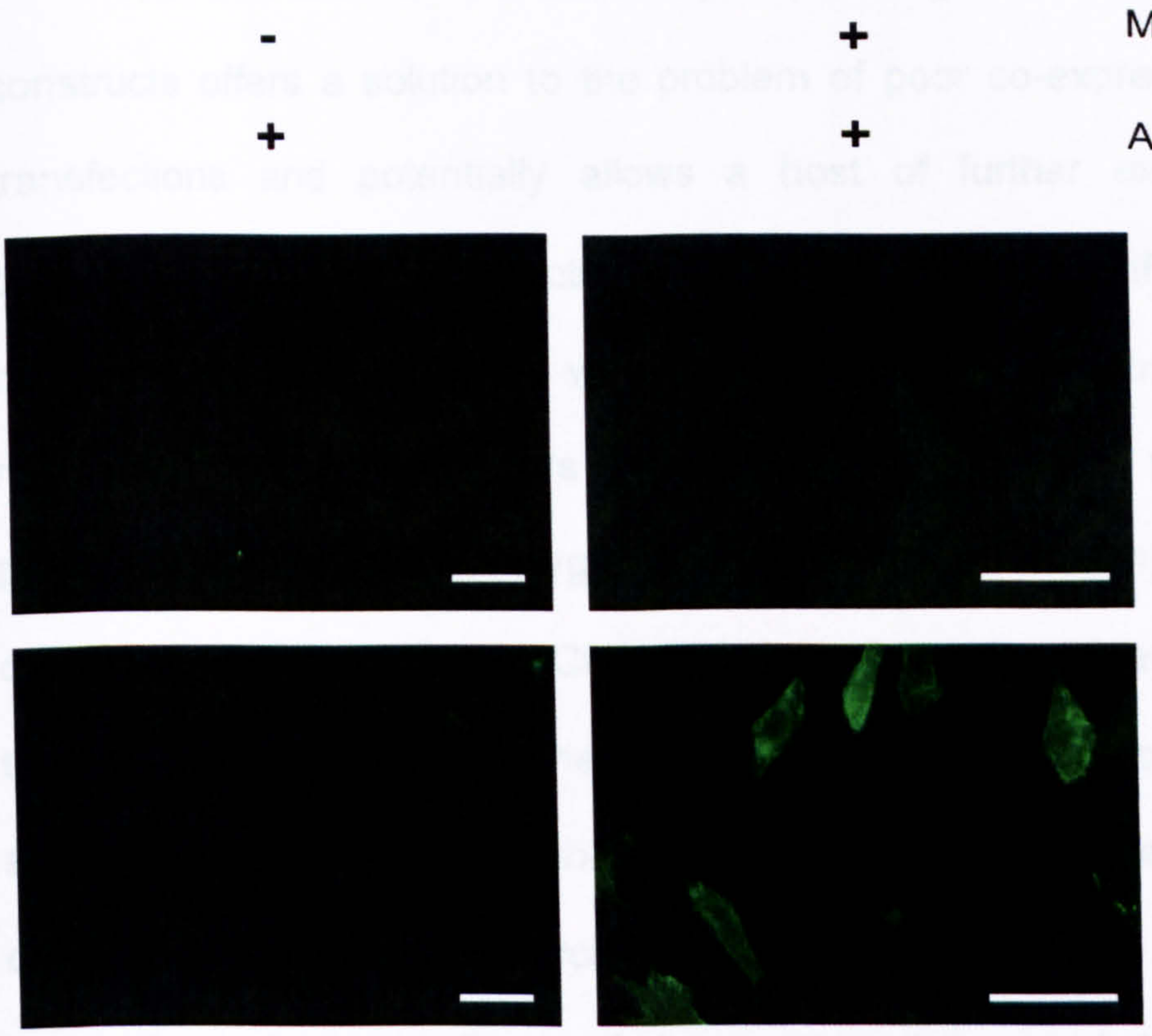


Figure 6.5.2
Transient expression of chFcR/L in CHO-K1 cells

Immunofluorescent anti-V5 staining of CHO-K1 cells grown on chamber slides transiently transfected with vectors encoding N-terminal tagged chFcR/L alone (top two images) or in concert with chFcRγ (bottom two images). Cells were incubated with mouse monoclonal anti-V5 antibodies (right two images) or buffer alone (left two images), followed by anti-mouse antibodies conjugated to FITC (Dako). Cells were analysed using an Axioscope fluorescence microscope and images collected using an Axiocam CCD and Axiovision software (Zeiss). Untransfected cells did not stain with anti-V5 (not shown). Scale = 100μm.

6.5.3 Stable expression of [FLAG]chFcRγ and [V5]chFcR/L

The selection of clones stably expressing the chFcR/L and chFcRγ constructs offers a solution to the problem of poor co-expression in transient transfections and potentially allows a host of further experiments to be performed. To allow dual selection of transfected cells, the chFcR/L and chFcRγ cassettes (Figure 6.5.1 C) were cloned into two commercially available mammalian expression vectors containing different antibiotic selection markers, pCDNA3 and pCDNA3.1/Hygro respectively (not shown). These vectors constitutively express from a CMV promoter and are practically identical except that pCDNA3 contains a gene which confers resistance to neomycin (or an analogue G418/Geneticin), whereas pCDNA3.1/Hygro contains a gene which confers resistance to hygromycin.

Several mammalian cell types commonly used to produce stable transfectants were screened for IgY binding. Whilst all were essentially negative, some cell types were found to have higher background fluorescence than others. A human embryonic kidney cell line, HEK 293, was chosen as it had low background, is only mildly adherent (so could be tapped off the culture flask surface easily for subsequent flow cytometry rather than requiring harsher methods of detachment, such as trypsin) and has been reported to be suitable for expression of chicken immune-type receptors in the literature (Viertlboeck *et al.*, 2004).

At the time of writing, stable transfectants have been selected which express [FLAG-chFcRγ] and [V5-chFcR/L] alone, as determined by FACS analysis (Figures 6.5.3 i and 6.5.3 ii), but a cell line expressing both concurrently has yet to be produced.

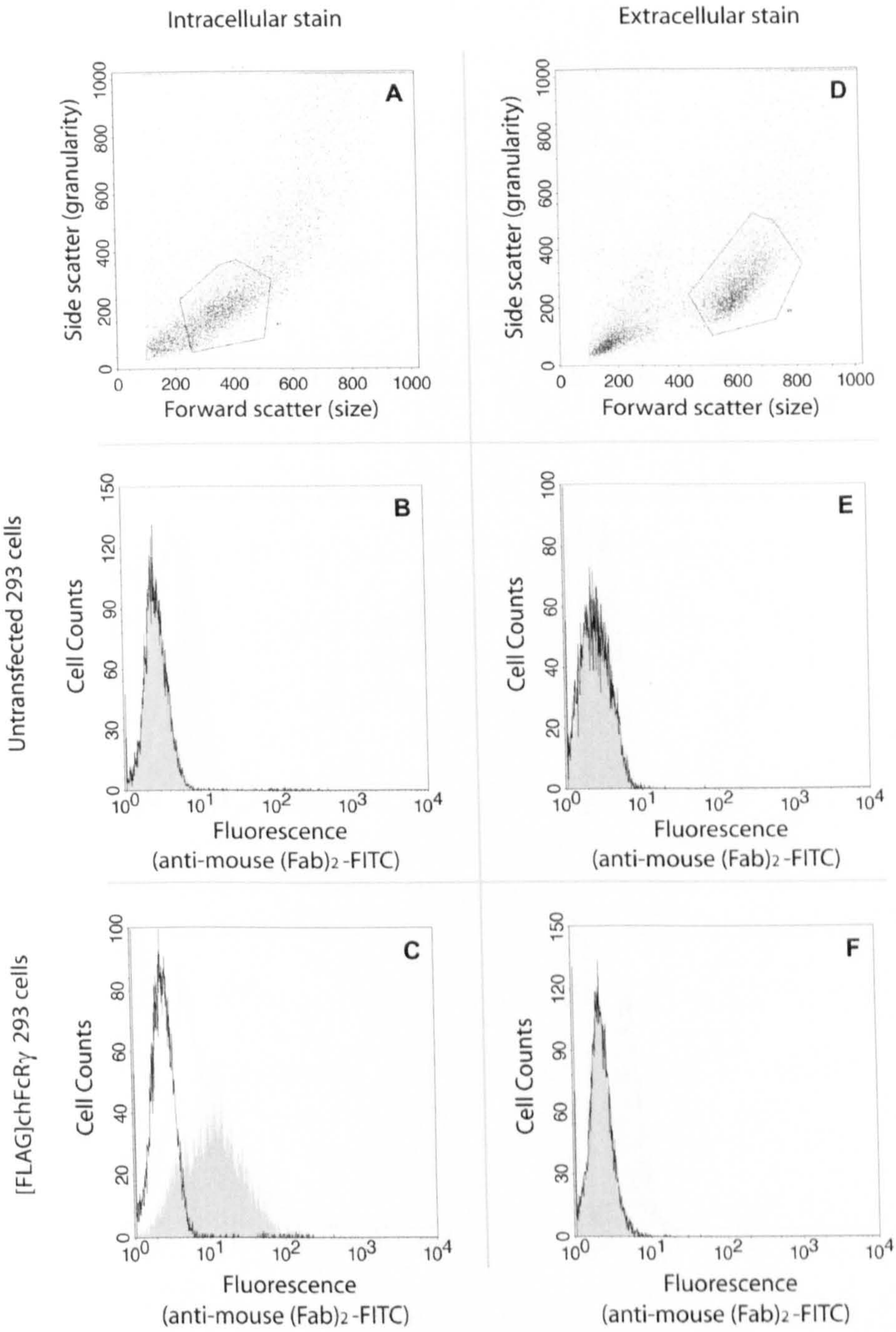


Figure 6.5.3 i
Stable expression of N-terminal FLAG tagged chFcRγ in HEK 293 cells

FACS profiles showing HEK 293 cells gated to exclude dead cells (A & D) following incubation with 1μg/ml mouse monoclonal anti-FLAG antibodies followed by 10μg/ml anti-mouse-FITC (filled peaks) or with buffer alone followed by 10μg/ml anti-mouse-FITC (unfilled peaks). Cells were fixed, washed and incubated in the presence (A, B & C) or absence (D, E & F) of 0.1% saponin for intracellular or extracellular staining respectively. The staining pattern of cells stably transfected with the vector for the [FLAG]chFcRγ construct (C & F) are shown in comparison with that of untransfected cells (B & E).

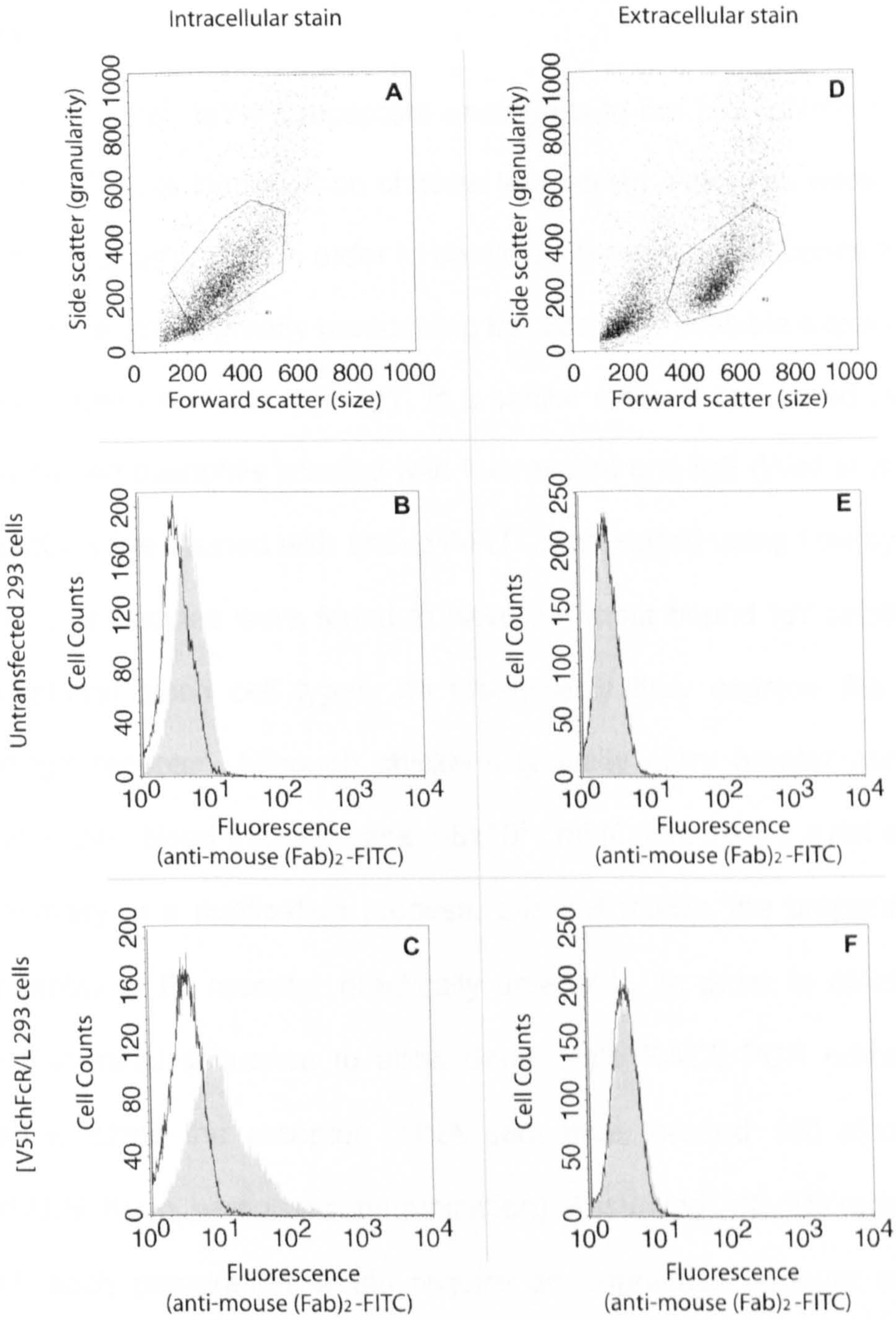


Figure 6.5.3 ii
Stable expression of N-terminal V5 tagged chFcR/L in HEK 293 cells

FACS profiles showing HEK 293 cells gated to exclude dead cells (A & D) following incubation with 1μg/ml mouse monoclonal anti-V5 antibodies followed by 10μg/ml anti-mouse-FITC (filled peaks) or with buffer alone followed by 10μg/ml anti-mouse-FITC (unfilled peaks). Cells were fixed, washed and incubated in the presence (A, B & C) or absence (D, E & F) of 0.1% saponin for intracellular or extracellular staining respectively. The staining pattern of cells stably transfected with the vector for the [V5]chFcR/L construct (C & F) are shown in comparison with that of untransfected cells (B & E).

6.6 Discussion

In this chapter, IgY-Fc receptors analogous to the high affinity receptors for IgG and IgE were identified on chicken leukocytes. Attempts were made to isolate a receptor as protein in order to obtain an N-terminal sequence for cDNA cloning, with the aim of greatly broadening the scope of possible experiments to characterise their interaction with IgY. In a similar method developed by Weil *et al.* to sort human basophils labelled with fluorescent anti-IgE (Weil *et al.*, 1983), avian PBMCs were stained with anti-IgY-FITC and sorted using flow cytometry. Basophilic granulocytes were found to have the most bound IgY relative to all other peripheral blood cell types, so presumably they express the greatest levels of IgY receptor. Although chickens typically carry greater numbers of basophils in their blood than humans, ~$8x10^5$ / ml (Cook, 1937), even assuming 100% recovery in a purification process, this still makes the preparation of a useful quantity of Fc receptor practically untenable. In order to obtain a long enough N-terminal sequence to allow degenerate RACE-PCR primers to be designed to clone the receptor cDNA sequence, around 100 picomoles is required (J.N. Keen, personal communication). Assuming 200,000 receptors per basophil, each preparation would require an impractical amount of chicken blood:

$$= \frac{(1x10^{-10}\text{moles} \times 6.022x10^{23})}{2.0x10^5\ \text{molecules/cell}} \times \frac{1}{8.2x10^5\ \text{basophils/ml}}$$

= 376 ml blood

IgY-Fc receptors were shown to be present on a chicken monocyte cell line, MQ-NCSU. Although the use of a cell line solves the practical issues with obtaining purified cells, the low level of receptor expression (1-2x10^4 molecules per cell) means that at least 3-6x10^9 cells (~5-10 litres of culture) would be required to obtain 100 pmol receptor. Small preparations were performed using affinity purification in an attempt to visualise receptor using SDS-PAGE. Unfortunately, too many contaminating proteins were found which could not be adequately removed by increased column wash volume or wash buffer ionic strength.

As protein-based approaches therefore seemed unfeasible with available resources, an *in silico* strategy was pursued to identify candidate Fc receptors in chicken EST databases and the chicken genome sequence, the first draft of which had recently been released (Hillier *et al.*, 2004). A single gene belonging to the Fc receptor family, *chFcR/L*, was identified by (translated nucleotide) BLAST searches using the amino acid sequences of mammalian FcεRI and all FcγRs, then cloned by RACE-PCR (Taylor *et al.*, 2007). Searches of subsequent drafts of the genome and all available bird EST libraries, including duck (Xia *et al.*, 2006), have not revealed any additional FcR-like genes. Fayngerts *et al.* cloned the same sequence and showed by Southern blot of restriction digested genomic DNA that this would appear to be the only sequence of its kind (Fayngerts *et al.*, 2007), although this is not conclusive. The presence of only one FcR family member in the chicken is rather surprising given that there are numerous homologues in amphibians, whose lineage diverged before the subdivision of birds and mammals (Guselnikov, 2004). Indeed, it would be unwise to assume there are no other homologues on the

basis of their absence from the database alone; many immune-related multigene families that consist of closely related sequences have proven difficult to fully sequence by the shotgun approach used for the chicken genome and thus show artefactual under-representation (Burt, 2005; Viertlboeck *et al.*, 2005). The IgY upsilon heavy chain gene is itself absent except for a single exon.

The *chFcR/L* gene and the encoded protein share several features characteristic of two important FcR subfamilies: the 'classical' Fc receptors, whose ligands are antibodies, and FCRL, whose ligands are currently unknown. As the genes in both of these families are essentially made up from the same pool of closely related exons, phylogenetic analysis was unable to conclusively place chFcR/L as a member of one or the other.

If just a single *FcR/FCRL* gene is present in the chicken, it would suggest that either all other genes have been lost in the avian lineage or that all mammalian members are derived from a chFcR/L-like ancestor – i.e. gene duplication events have occurred in the mammalian lineage, but not the avian lineage. Several FCRL members contain a domain 'type' which does not occur in chFcR/L (coloured green in figure 6.3.6). This type has either been lost in the avian lineage or is the result of divergence from one of the other domain subfamilies. Perhaps the most plausible explanation is a combination of both of these; the FcR/FCRL subdivision occurred in the mammalian lineage following the split from the avian lineage (~300 million years ago), but (unlike the IgY/IgG/IgE situation) part or all of the ancestral gene or genes have been lost in modern day birds.

Comparison of binding domains in FcR with the most closely related domain in chFcR/L is of limited use to predict IgY binding potential. In order to determine whether chFcR/L is an IgY-Fc receptor it is therefore necessary to express the sequence and assay IgY binding directly. When transiently expressed in CHO cells, surface translocation of chFcR/L was found to require the co-expression of an accessory subunit, the chicken orthologue of FcRγ, to allow translocation to the cell surface. In mammals, several FcR require this subunit in order to be expressed on the cell surface and/or acquire signalling potential, unlike any known FCRL, which carry their own (functional) cytoplasmic signalling motifs (Davis *et al.*, 2002; Leu *et al.*, 2005). Co-expression was, however, found to be too inefficient to allow adequate characterisation of IgY-binding potential. To overcome this problem, steps were made toward the creation of a stable cell line co-expressing sufficient levels of chFcR/L and chFcRγ, which should allow a conclusive appraisal of IgY-binding.

Should chFcR/L prove to be an IgY-Fc receptor, it will be interesting to discover whether chFcR/L ΔEC_2 is also capable of binding, as this splice variant lacks the domain homologous to the mammalian FcR binding domains. The kinetics of IgY binding could then be investigated by cell binding assay and compared to that of the monocyte IgY-Fc receptor, which may be the same molecule. Preparation of anti-chFcR/L specific antibodies would allow this issue to be resolved.

Should chFcR/L prove not to be an IgY-Fc receptor, the findings described in this chapter allow a new approach to be taken; expression libraries could be created using cDNA derived from FACS sorted chicken basophils and MQ-NCSU monocytes, then transfected into the HEK 293 cells stably

expressing chFcRγ, in order to increase the chance of discovering receptors whose surface expression requires subunit association. The cells would then be screened for the ability to bind IgY, and cDNA isolated from positive hits. This technique was used to clone and sequence the human high affinity receptor for IgG, FcγRI.

Chapter 7

Functional Characterisation of IgY-Fc Receptor Interaction on Chicken Monocytes

7.1 Overview

Previous chapters detail the design, expression and purification of a series of recombinant IgY-Fc mutants. Although a recombinant soluble IgY-Fc receptor has yet to be produced, IgY-Fc receptors are expressed by a monocyte cell line, MQ-NCSU, indicated by IgY-dependent effector functions (Qureshi *et al.*, 1990) and monomeric IgY binding using flow cytometry (see chapter 6, section 6.2.2). These cells therefore provide a means to make some preliminary studies on various properties of an IgY-Fc receptor, including the interaction with IgY-Fc.

For comparative purposes, there are a host of published cell binding data for IgG and IgE, including interactions with mammalian monocytes specifically (McCool *et al.*, 1985; Raychaudhuri *et al.*, 1985; Unkeless and Eisen, 1975). The effects of various structural features on the kinetics of IgE binding to FcεRIα have been investigated using surface plasmon resonance (SPR) or analytical ultracentrifuge (AUC) techniques (Henry *et al.*, 1997; Hunt *et al.*, 2005; McDonnell *et al.*, 2001). There are often discrepancies between data obtained using these techniques and data obtained using cell binding assays. Comparisons between data collected using the same methodology are

therefore the most reliable, although for completeness, it is nevertheless important to consider all related findings.

The present chapter details initial studies into the effects of the various structural differences between the IgY-Fc mutants from a functional viewpoint. The factors investigated influence the Fc receptor interactions of IgG and IgE in markedly different ways, and thus characterisation of their role in IgY biology was an important aim.

7.2 Specificity of IgY and a chicken monocyte Fc receptor

Cross-species reactivity was investigated by attempting to bind (1) chicken IgY to cells expressing human Fc receptors for IgG or IgE and (2) human IgG or IgE-Fc to chicken monocytes (Figure 7.2).

Although rat IgE binds to human FcεRI (Conrad *et al.*, 1983), human IgE will not bind to rat FcεRI (Froese, 1980). Human IgG, by contrast, is more promiscuous and all but one subclass can bind to mouse FcγRI (Haeffner-Cavaillon *et al.*, 1979a; Haeffner-Cavaillon *et al.*, 1979b). Both chicken antibody and Fc receptor were found to be species specific. Neither IgG nor IgE-Fc were able to bind to MQ-NCSU cells and chicken IgY did not bind to cells expressing FcεRI or FcγRI. Over the course of the investigation, IgG antibodies from mouse, rabbit and goat were also tested and found to be non-reactive (not shown).

Lack of cross-species reactivity is likely to be the result of poor sequence identity in the antibody and Fc receptor binding sites due to the relatively large evolutionary distance between birds and mammals. This diversity may be more

due to differences in the (immune-type) receptors. Sufficient homology in certain surface loops exists between human IgG and chicken IgY to allow cross-reactivity with an IgY receptor that mediates transport into the yolk when human IgG is injected intravenously (Morrison *et al.*, 2002), although this is inconsistent with the finding that the recently cloned yolk sac IgY receptor does not bind human IgG or IgE in an SPR assay (West *et al.*, 2004).

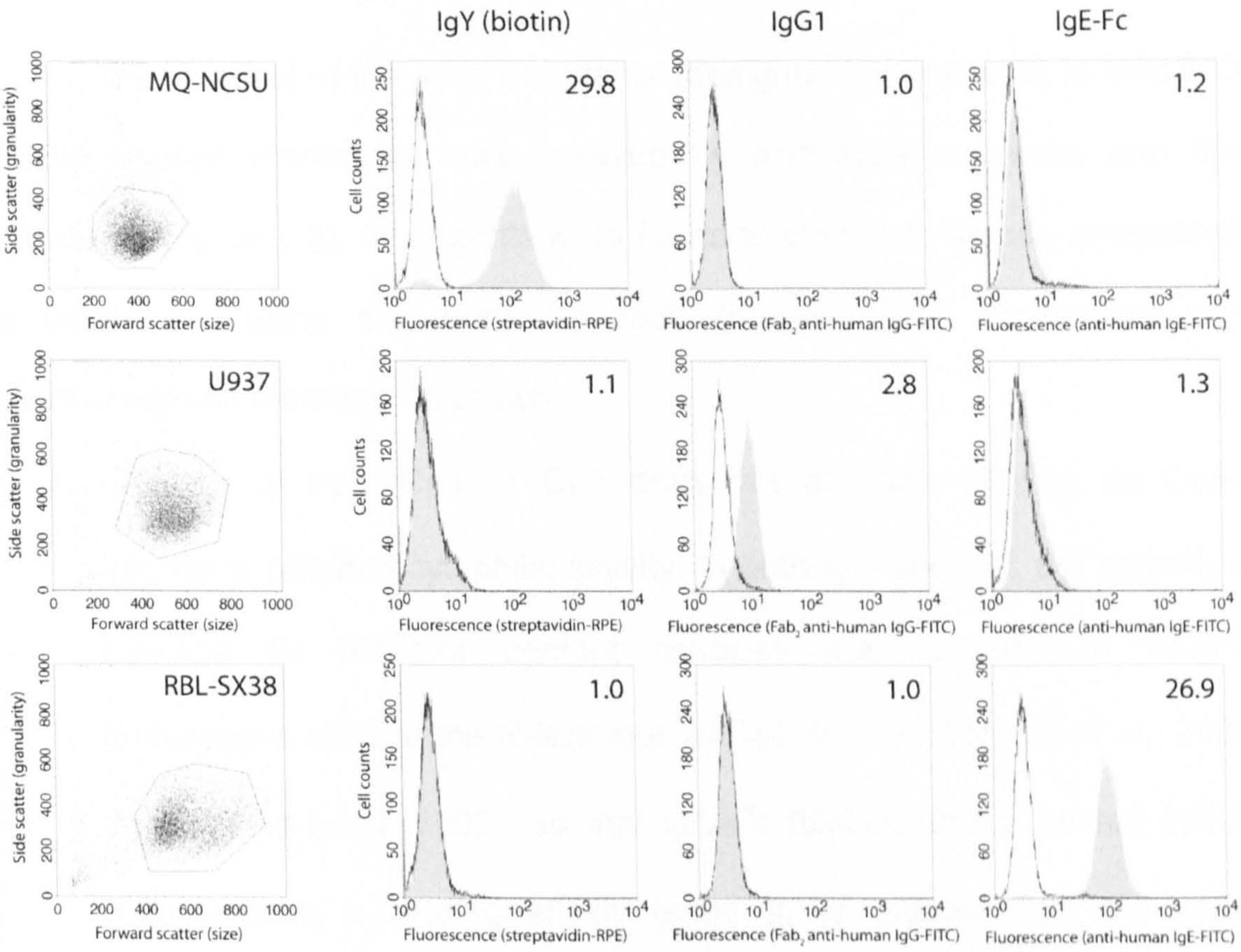


Figure 7.2
Specificity of IgY for chicken monocyte Fc receptors

Flow cytometry (FACS) profiles showing binding of secondary fluorescent conjugates to three different cell lines (rows) following incubation with chicken IgY, human IgG1 and recombinant human IgE-Fc (columns). MQ-NCSU are chicken monocytes, U937 are human monocytes and RBL-SX38 are rat basophils expressing the human high affinity receptor for IgE. Values shown are ratios of mean fluorescence intensity of cells incubated with tested protein then secondary conjugate (filled peaks) over that of control cells incubated with conjugate alone (unfilled peaks). Cell types were gated as marked in the dot plots.

7.3 Fc receptor binding potential of IgY-Fc mutants

The potential of the IgY-Fc mutants (designed in chapter 4) to bind MQ-NCSU chicken monocytes was assayed by immunofluorescence and flow cytometry (Figure 7.3). All mutants were found to bind, with signals comparable to whole IgY using the same antibody (Figure 6.2.2). Some important conclusions can therefore be drawn:

1. Absence of Fab, Cυ1 or Cυ2 does not abrogate binding as Cυ3-4 fragments retained cytophilic activity. In both IgG and IgE, the majority of classical Fc receptor contact residues are clustered in roughly homologous sites at the N-terminus of Cγ2 or Cε3 (Garman *et al.*, 2000; Jefferis and Lund, 2002), so that IgG-Fc (Cγ2-3) and truncated IgE-Fc (Cε3-4) have a binding affinity equal to or greater than the whole antibody (Keown *et al.*, 1997; Keown *et al.*, 1995; Raychaudhuri *et al.*, 1985). Conservation of the approximate location of the receptor binding site in IgY (Cυ3) seems likely and is consistent with the observed binding potential of Cυ3-4 fragments.
2. An inter-heavy chain disulfide bond is not an absolute requirement for binding as the Cυ3-4 [C340S C347S] mutant was able to bind to MQ-NCSU monocytes. Fc fragments of IgG and IgE also retain the ability to bind to their receptors when interchain disulfides are absent (Basu *et al.*, 1993; Helm *et al.*, 1991; Lund *et al.*, 1993), albeit with lower affinity (Hunt *et al.*, 2005; McCool *et al.*, 1985).
3. Absence of glycosylation at one or both sites (N308 and N407) does not abrogate binding as the Cυ2-4 [N308Q] mutant and the aglycosylated

Cυ2-4 [N308Q N407Q] mutant were both able to bind to MQ-NCSU monocytes. This is perhaps the most unexpected finding as it highlights a significant difference between the role of carbohydrate in IgY and IgG. Deglycosylated IgG is unable to engage Fc receptors (Walker *et al.*, 1989), whereas IgE stripped of carbohydrate is still able to bind with high affinity (Hunt *et al.*, 2005).

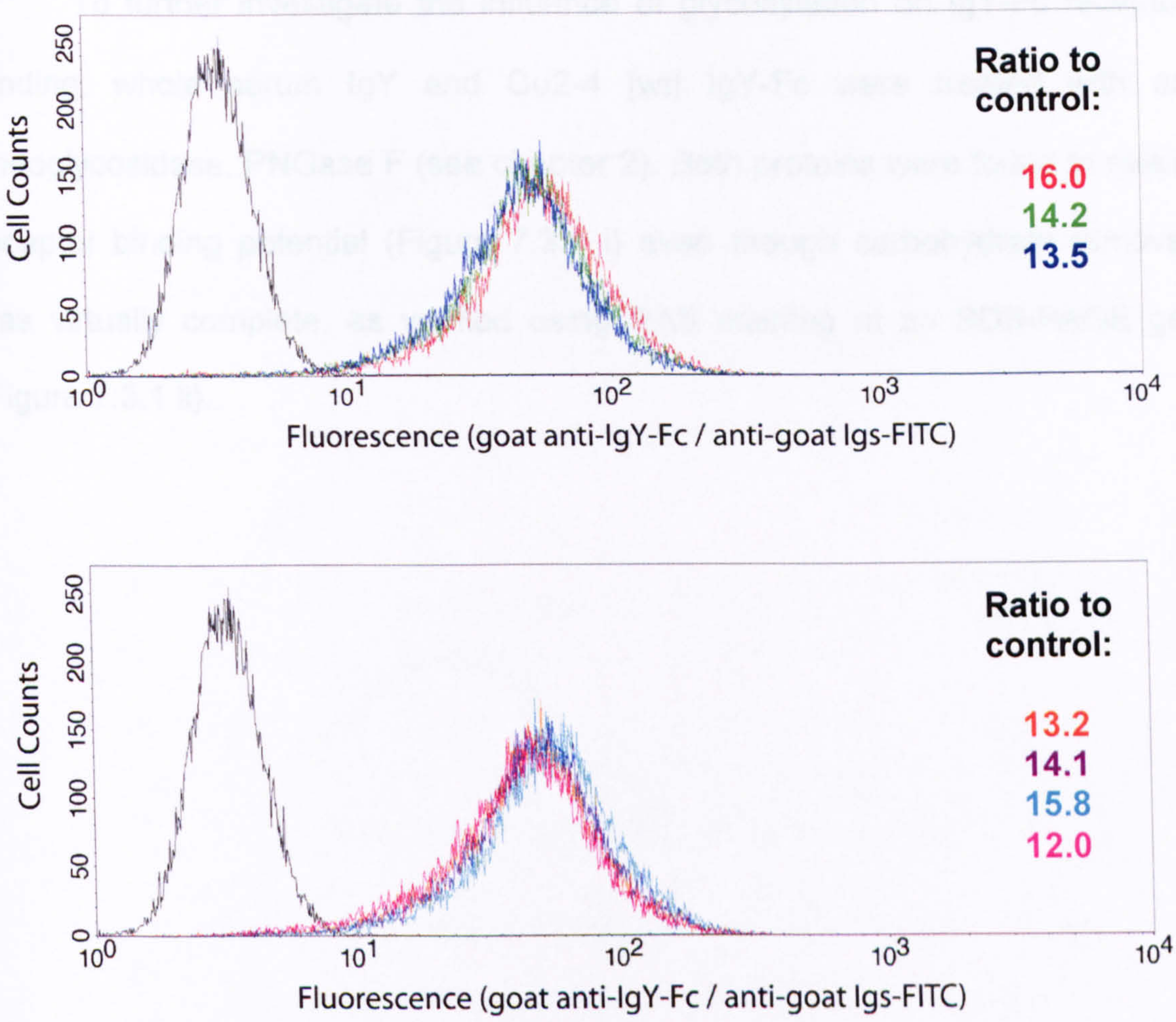


Figure 7.3
Receptor binding potential of IgY-Fc mutants to MQ-NCSU chicken monocytes

Flow cytometry (FACS) profiles showing binding of antibodies specific for IgY-Fc followed by secondary fluorescent conjugates to MQ-NCSU cells. Cells were first incubated with Cυ2-4 mutants (top), [wt] (red), [N308Q] (green), [N308Q N407Q] (blue) or Cυ3-4 mutants (bottom) [wt] (orange), [C340S] (purple), [C347S] (cyan), [C340S C347S] (magenta), or buffer alone (black). Values shown are ratios of mean fluorescence intensity of cells incubated with IgY-Fc then secondary antibody/conjugate over that of control cells incubated with buffer alone then secondary antibody/conjugate.

7.3.1 Effect of glycosylation of IgY-Fc on monocyte receptor binding

To further investigate the influence of glycosylation on IgY-Fc receptor binding, whole serum IgY and Cυ2-4 [wt] IgY-Fc were treated with an endoglycosidase, PNGase F (see chapter 2). Both proteins were found to retain receptor binding potential (Figure 7.3.1 i) even though carbohydrate removal was virtually complete, as verified using PAS staining of an SDS-PAGE gel (Figure 7.3.1 ii).

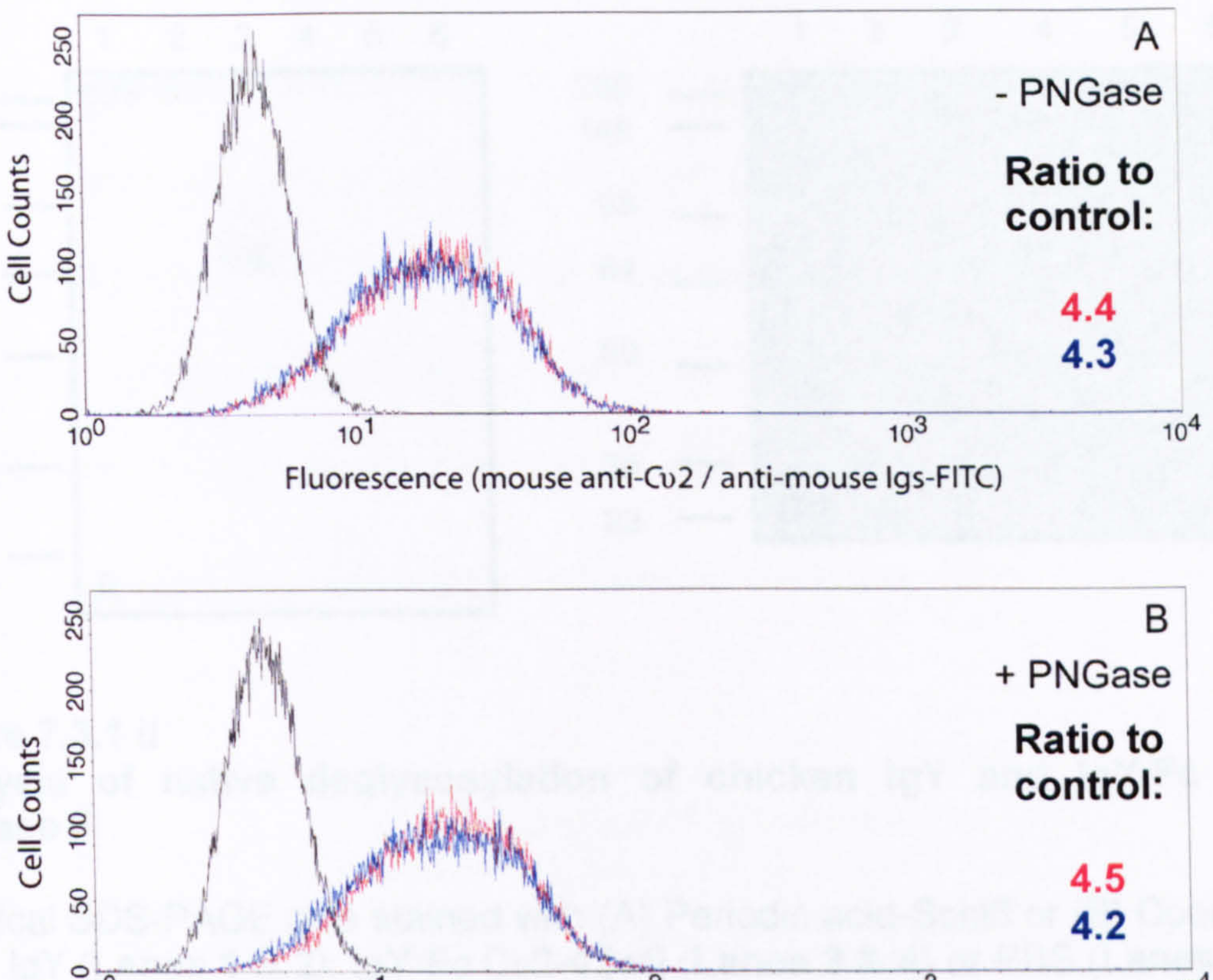


Figure 7.3 i
Receptor binding potential of chicken IgY and human IgG1 following treatment with PNGase

Flow cytometry (FACS) profiles showing binding of chicken serum IgY or IgY-Fc Cu2-4 [wt] to MQ-NCSU cells (A) before and (B) after incubation with PNGase F (NEB) for 3 days at 37°C. Cells were incubated with IgY (red) or IgY-Fc (blue) followed by antibodies specific for Cυ2 then secondary fluorescent conjugates. For comparison, a profile of human IgG1 binding to U937 human monocytes is shown (C), before (blue) and after (red) the same PNGase F treatment. Values shown are ratios of mean fluorescence intensity of cells incubated with tested protein then secondary antibody/conjugate over that of control cells incubated with buffer then secondary antibody/conjugate alone (black trace in all profiles).

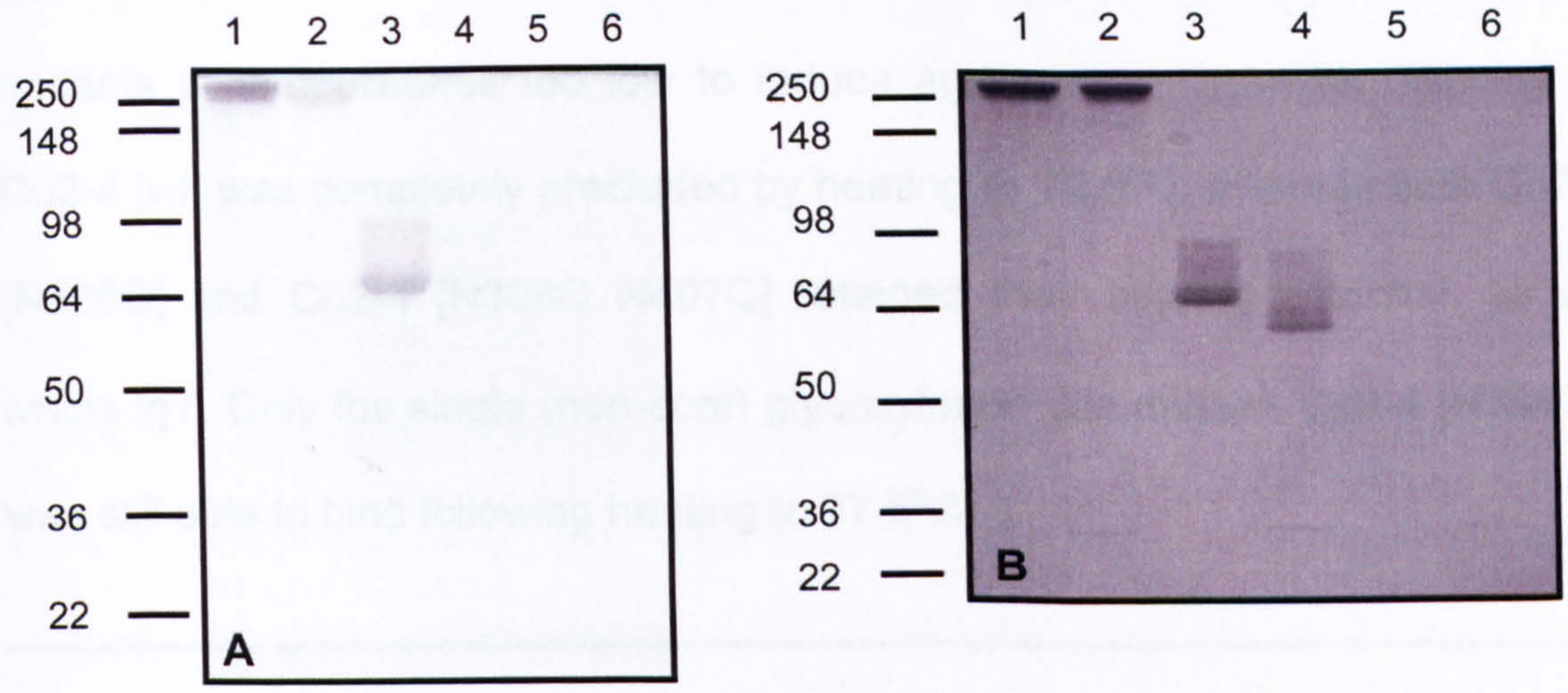


Figure 7.3.1 ii
Analysis of native deglycosylation of chicken IgY and IgY-Fc using PNGase F

Identical SDS-PAGE gels stained with (A) Periodic acid-Schiff or (B) Coomassie blue. IgY (**Lanes 1 & 2**), IgY-Fc Cυ2-4 [wt] (**Lanes 3 & 4**) or PBS (**Lanes 5 & 6**) were incubated for 3 days at 37°C in non-denaturing deglycosylation buffer without (**Lanes 1, 3 & 5**) or with (**Lanes 2, 4 & 6**) PNGase F (NEB). Molecular weight is shown in kDa. PNGase F enzyme has a molecular weight of ~36kDa; bands approximately equivalent to this can be seen in (B), lanes 2, 4 & 6.

7.3.2 Effect of IgY-Fc glycosylation on heat-induced abrogation of receptor binding potential

Whole serum IgY, IgY-Fc Cυ2-4 [wt] and the Cυ2-4 glycosylation site mutants were heated for four hours at a series of temperatures around their melting temperatures (see section 5.4.5) using a PCR block. Proteins were then cooled to 4°C and incubated with MQ-NCSU monocytes. Binding was assayed using immunofluorescence and flow cytometry (Table 7.3.2).

At 82.5°C, all four molecules exhibited loss of receptor binding, which is likely the result of unfolding or occlusion of binding sites due to thermally-induced aggregation, consistent with data obtained using DLS (Figure 5.4.4). A

significant difference was seen between Cυ2-4 [wt] and the glycosylation site mutants at temperatures too low to induce aggregation, however. Binding of Cυ2-4 [wt] was completely precluded by heating to 72.5°C, whereas both Cυ2-4 [N308Q] and Cυ2-4 [N308Q N407Q] retained their binding potential, as did whole IgY. Only the single (non-core) glycosylation site mutant, Cυ2-4 [N308Q], was still able to bind following heating to 77.5°C.

		Temperature of ligand pre-treatment (°C)				
		4	67.5	72.5	77.5	82.5
	Whole IgY	4.83	3.67	3.00	1.32	1.06
IgY-Fc (Cυ2-4)	wt	4.21	3.66	1.21	1.14	1.11
	N308Q	3.84	3.66	3.42	2.74	1.16
	N308Q N407Q	3.52	2.83	2.37	1.36	1.11

Table 7.3.2
Chicken monocyte binding potential of heat treated IgY and IgY-Fc glycosylation site mutants

Whole serum IgY and recombinant IgY-Fc mutants were heated to the temperatures shown at 0.1mg/ml then cooled to 4°C and incubated with MQ-NCSU cells at 0.01mg/ml. Cells were then incubated with mouse monoclonal anti-Cυ2 specific antibodies followed anti-mouse FITC, then assayed by FACS. Values shown are ratios of mean fluorescence intensity of cells incubated with tested protein then secondary antibody/conjugate over that of control cells incubated with buffer then secondary antibody/conjugate alone.

7.4 Determination of kinetics of IgY and IgY-Fc binding to chicken monocytes

Cell binding assays were performed to evaluate the kinetics of IgY and recombinant IgY-Fc binding to MQ-NCSU monocytes. A well established method is to monitor binding using radiolabelled ('hot') ligand. Fluorescent labelling measured using flow cytometry has been shown to be effective for relative measurement of antibody:antigen affinities (Geuijen *et al.*, 2005) and was initially investigated as a safer alternative.

IgY labelled directly with fluorescein isothiocyanate (FITC) was found to bind to MQ-NCSU cells, but the fluorescence signal was insufficient for kinetic studies (not shown). To solve this, IgY was biotinylated and the signal amplified using streptavidin conjugated to phycoerythrin (RPE).

Although this provided a reasonable signal for IgY (a dissociation experiment is shown in appendix 1), the signals from biotinylated versions of the IgY-Fc mutants were too low to allow accurate measurement of bound material (not shown), presumably due to the smaller number of lysine residues available for reaction with biotin in the Fc fragment compared with the whole antibody. Radiolabelling was therefore deemed to be more suitable for studies involving the IgY-Fc mutants. IgY or recombinant IgY-Fc were labelled with radioactive iodine-125 (^{125}I) using chloramine T (McConahey and Dixon, 1980). Cells saturated with either whole antibody or IgY-Fc were found to have radioactivity levels well within the sensitivity of the automatic gamma counter used, a Wizard 1480 (Perkin Elmer).

7.4.1 Determination of receptor numbers on MQ-NCSU chicken monocytes

^{125}I incorporation was determined by measurement of the mean counts per minute (cpm) of a known concentration of labelled IgY. IgY (and all IgY-Fc mutants) were found to have a specific activity of ~1x10^9 cpm/mg. 10^6 MQ-NCSU cells cultured in IgY-free media were saturated with hot IgY, separated from unbound free ligand and their mean cpm measured. Assuming 1:1 binding stoichiometry, the mean number of bound IgY molecules equal to the mean number of receptor sites was calculated to be 15,000 ± 5,000. This is comparable to the number of FcγRI molecules reported on primary human monocytes, 4,300 – 45,000 (Jungi and Hafner, 1986; Karagiannis *et al.*, 2003), and a human monocyte cell line (U937), which express 18,000 receptors per cell (Anderson and Abraham, 1980).

7.4.2 Binding kinetics of radioiodinated IgY and recombinant IgY-Fc mutants to chicken monocytes

The association rate constant (on-rate or k_{+1}) and dissociation rate constant (off-rate or k_{-1}) were determined for IgY and all recombinant IgY-Fc mutants binding to MQ-NCSU monocytes by experiments monitoring the change in bound ^{125}I-labelled ligand over time. Briefly, for association experiments, MQ-NCSU cells were incubated with radiolabelled ligand and cells were removed at regular intervals for gamma counting. For dissociation experiments, cells were first saturated with hot ligand then incubated with an excess of unlabelled 'cold' ligand in order to prevent re-binding of dissociated material. The rate constants were determined by non-linear regression analysis

of the scintillation counts per minute (proportional to the concentration of bound ligand) as a function of time (see chapter 2 for equations used).

Figures of plots obtained with IgY (Figure 7.4.2 i) and IgY-Fc Cυ2-4 [wt] (Figure 7.4.2 ii) are shown as exemplars; figures for the remaining IgY-Fc mutants can be found in appendix 1. The results collected so far are summarised in table 7.4.2 iii. Some initial observations can be made from the data collected so far, although it should be noted that these results are preliminary as more replicates and additional experiments are required to reduce errors and increase statistical significance. The limitations and caveats of these assays are discussed in section 7.5.

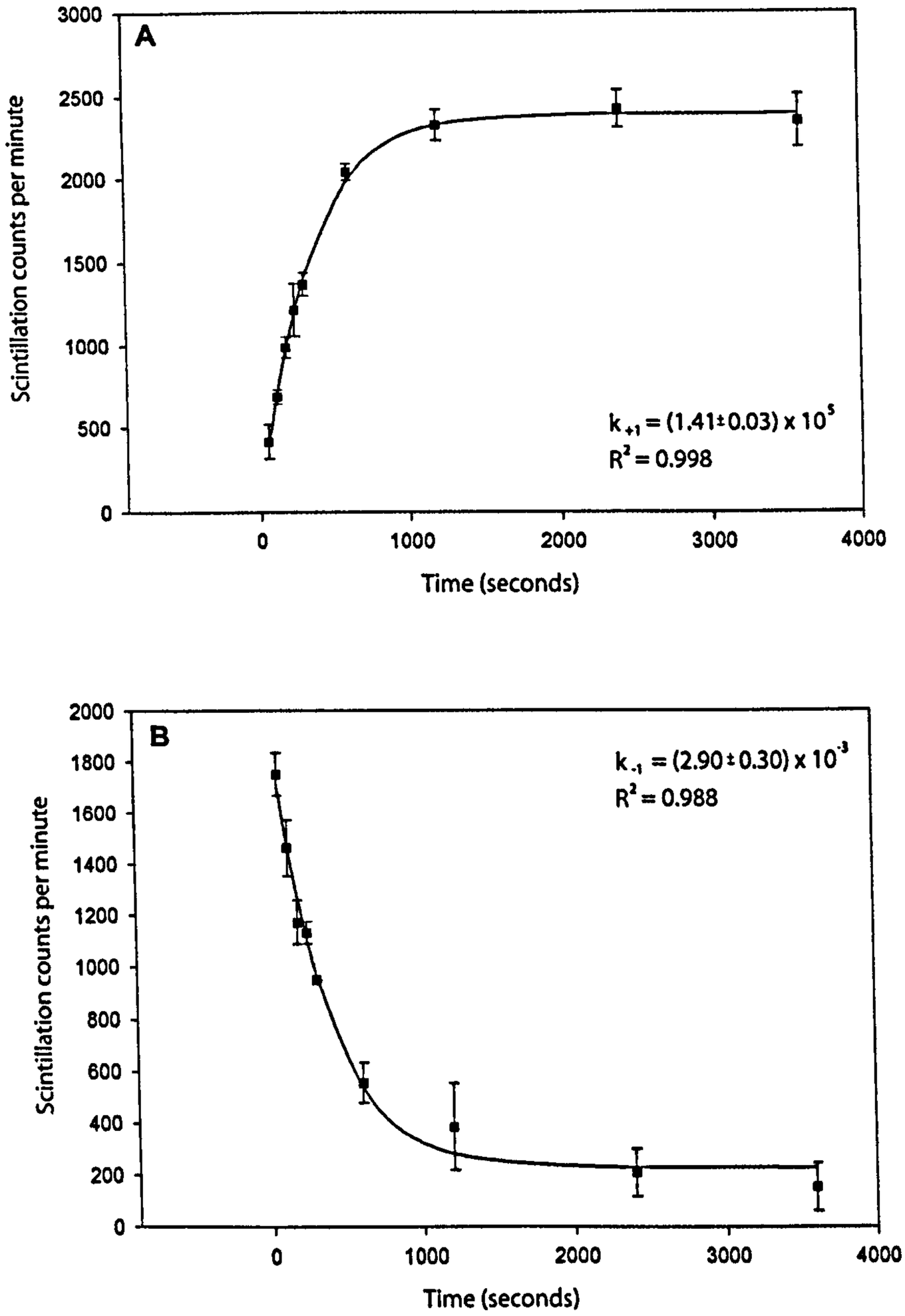
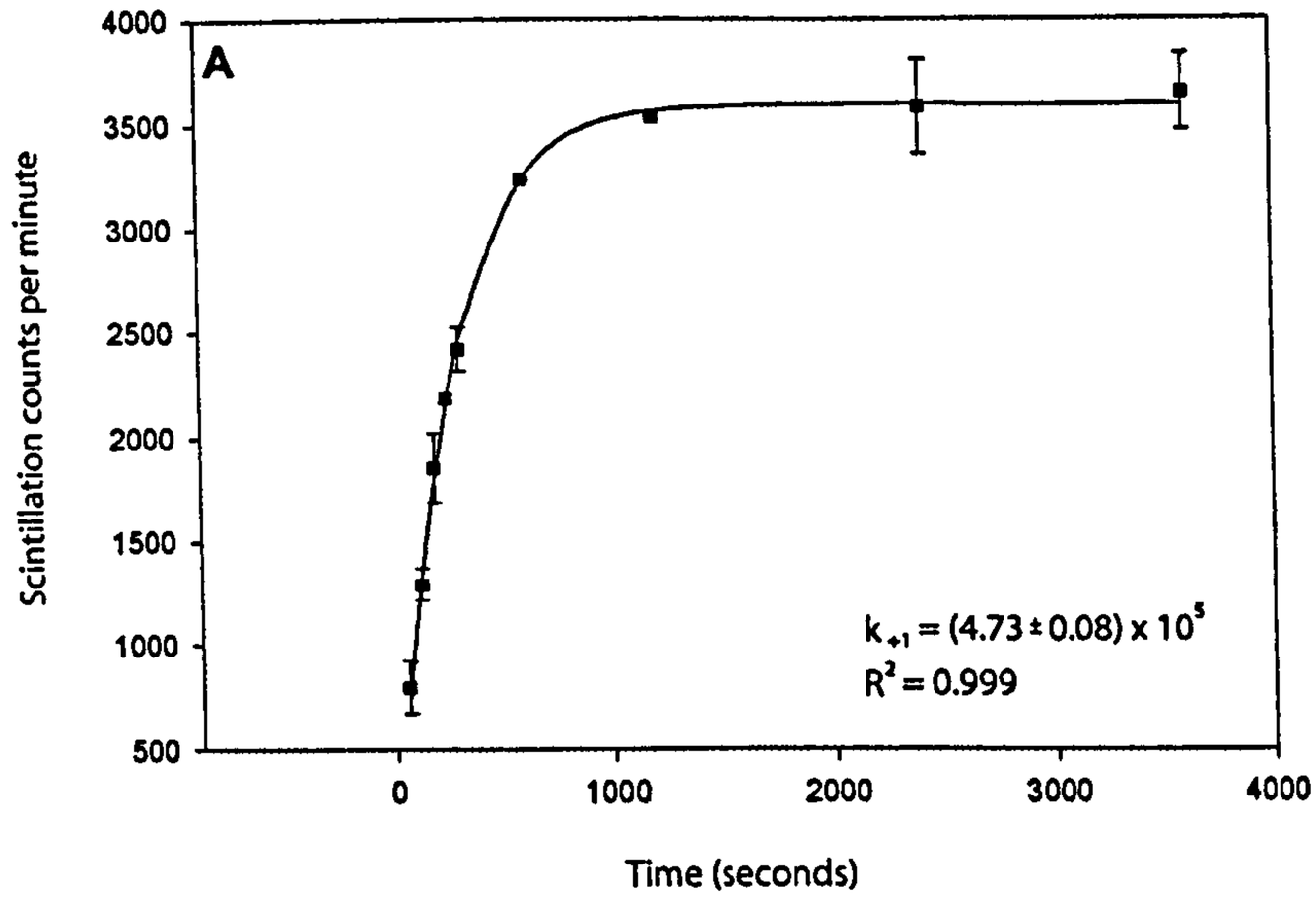


Figure 7.4.2 i
Kinetics of IgY binding to chicken monocytes

Cell binding assays showing experiments used to determine (A) association rate constant (k_{+1}) and (B) dissociation rate constant (k_{-1}) for chicken serum IgY binding to MQ-NCSU cells. Assays were performed in duplicate and data are shown as mean ± s.e.m.
In (A) cells were incubated in 10nM ^{125}I-labelled ligand. In (B) cells were first saturated with ^{125}I-labelled ligand then washed and incubated with excess unlabelled ligand. In both experiments, 10^6 cells were removed at the times shown, spun through phthalate oil and their radioactivity measured using an automatic gamma counter. Regression analysis was used to fit the data to non-linear models (see chapter 2). The R^2 value provides a measure of how well the models fit the data, with 1.0 being a perfect fit.



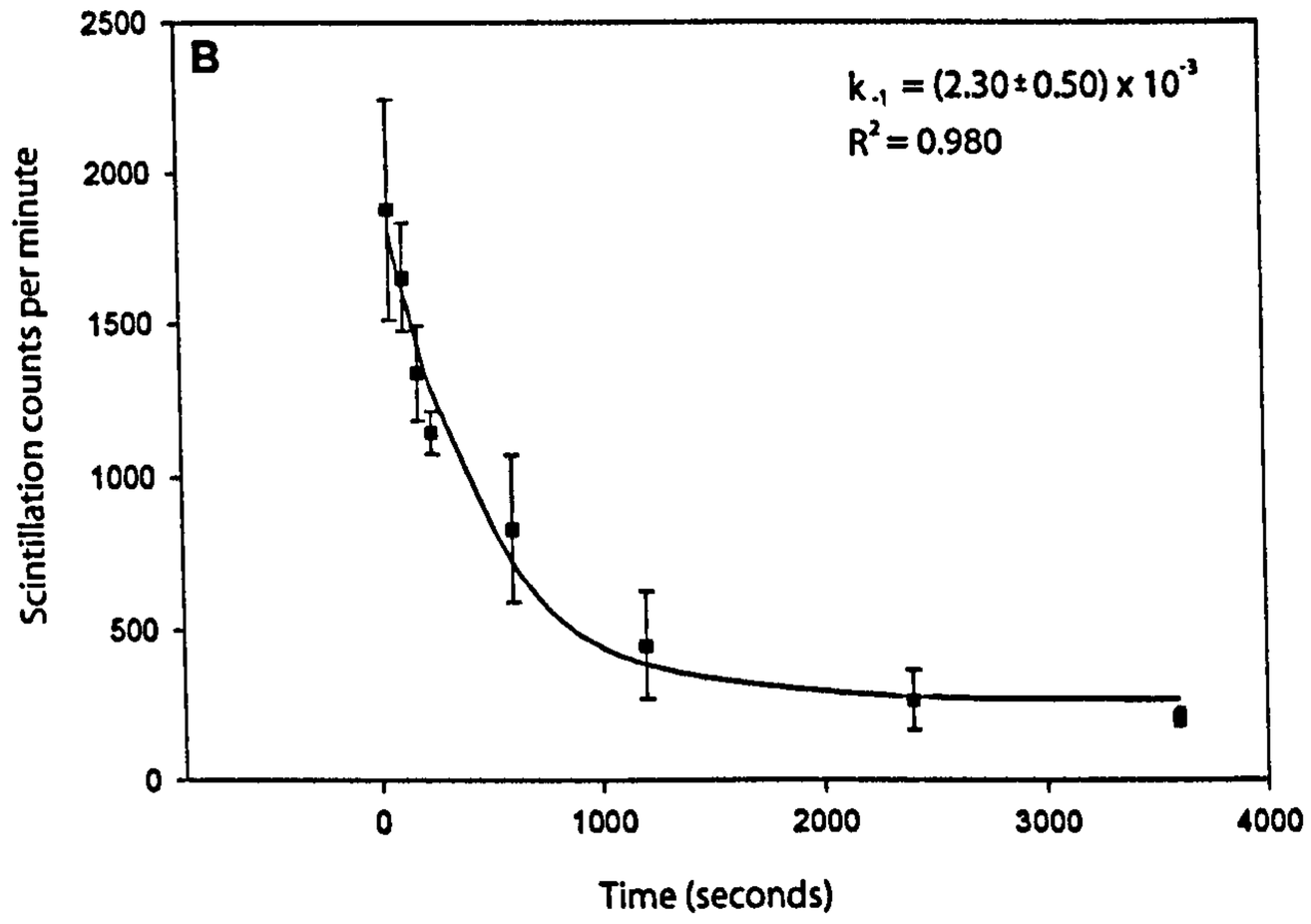


Figure 7.4.2 ii
Kinetics of IgY-Fc Cυ2-4 [wt] binding to chicken monocytes

Cell binding assays showing experiments used to determine (A) association rate constant (k_{+1}) and (B) dissociation rate constant (k_{-1}) for recombinant IgY-Fc Cυ2-4 [wt] binding to MQ-NCSU cells. Assays were performed in duplicate and data are shown as mean ± s.e.m.
In (A) cells were incubated in 10nM ^{125}I-labelled ligand. In (B) cells were first saturated with ^{125}I-labelled ligand then washed and incubated with excess unlabelled ligand. In both experiments, 10^6 cells were removed at the times shown, spun through phthalate oil and their radioactivity measured using an automatic gamma counter. Regression analysis was used to fit the data to non-linear models (see chapter 2). The R^2 value provides a measure of how well the models fit the data, with 1.0 being a perfect fit.

Protein		k_{+1} (M^{-1} s^{-1})	k_{-1} (s^{-1})	K_A (M^{-1})
Whole IgY		(1.41 ± 0.03) x 10^5	(2.90 ± 0.30) x 10^{-3}	4.9 x 10^7
IgY-Fc Cυ2-4	wt	(4.73 ± 0.08) x 10^5	(2.30 ± 0.50) x 10^{-3}	2.1 x 10^8
	N308Q	(2.38 ± 0.07) x 10^5	(3.60 ± 0.40) x 10^{-3}	6.6 x 10^7
	N308Q N407Q	(8.26 ± 1.02) x 10^4	(6.20 ± 0.80) x 10^{-3}	1.3 x 10^7
IgY-Fc Cυ3-4	wt	(4.26 ± 0.44) x 10^5	(8.10 ± 0.40) x 10^{-3}	5.3 x 10^7
	C340S	(2.18 ± 0.04) x 10^5	(5.20 ± 1.10) x 10^{-3}	4.2 x 10^7
	C347S	(2.00 ± 0.27) x 10^5	(2.17 ± 0.42) x 10^{-2}	0.92 x 10^7
	C340S C347S	(4.14 ± 0.52) x 10^5	(7.40 ± 1.70) x 10^{-3}	5.6 x 10^7

Table 7.4.2 iii
Summary of IgY and IgY-Fc monocyte interaction kinetics derived from cell binding assays

On-rates (k_{+1}) and off-rates (k_{-1}) derived from regression analysis of MQ-NCSU cell binding data using ^{125}I-labelled IgY and recombinant IgY-Fc mutants. Values are means of duplicate experiments shown ± standard error, determined using SigmaPlot 3.0 (SPSS).

The kinetics of the chicken IgY (complete antibody) binding to chicken monocytes were compared with the results of homologous interactions reported in the literature. Although there is some variation in the published values, the on-rate of IgY binding to monocytes (≈ 1.4×10^5 $M^{-1}s^{-1}$) was found to be similar to human IgG binding to monocytes, 1.9×10^5 $M^{-1}s^{-1}$ and 2.1×10^5 $M^{-1}s^{-1}$ (Raychaudhuri *et al.*, 1985; Shopes *et al.*, 1990). However, the off-rates determined in these studies were 20-50 fold slower (5.5×10^{-5} s^{-1} and 1.1×10^{-4} s^{-1}) than the dissociation of IgY (2.9×10^{-3} s^{-1}). Despite this difference, the binding parameters of IgY are within the range of mammalian IgGs; mouse IgG2a (the principal serum isotype) binds to P388D mouse monocytes with an on-rate of ~10^5 $M^{-1}s^{-1}$ and an off-rate of 3.5×10^{-3} s^{-1} (Unkeless and Eisen, 1975).

The IgY-Fc Cυ2-4 [wt] fragment was found to have a four-fold higher affinity than the complete IgY, predominantly due to a faster on-rate (4.7×10^5 $M^{-1}s^{-1}$). An almost identical difference (i.e. higher affinity for Fc compared with whole antibody) has been reported for the high-affinity receptor interactions of both human IgG and IgG-Fc (Raychaudhuri *et al.*, 1985) and human IgE and IgE-Fc (Cε2-4) using cell binding assays (Young *et al.*, 1995) as well as SPR (Henry *et al.*, 1997). As the binding sites in all three isotypes map to the Fc region, removal of the Fab moieties probably results in increased accessibility, which would increase the probability of ligand-receptor interactions in solution. Interestingly, this is not the case for the interaction between IgE and its low affinity receptor, CD23 (a C-type lectin, not an FcR family member). The IgE-Fc Cε2-4 fragment reportedly binds to CD23 with ten-fold lower affinity than the whole antibody (McDonnell *et al.*, 2001).

Although glycosylation was found not to be an absolute requirement for receptor binding in section 7.3, the absence of putative core glycosylation from Cυ2-4 fragment was found to have a significant effect on the receptor binding affinity. For the Cυ2-4 [N308Q] mutant, only minor differences were observed (k_{+1} = 2.4 x 10^5 $M^{-1}s^{-1}$, k_{-1} = 3.6 x 10^{-3} s^{-1}) compared to the wild type Cυ2-4 (k_{+1} = 4.7 x 10^5 $M^{-1}s^{-1}$, k_{-1} = 2.3 x 10^{-3} s^{-1}). For the aglycosylated Cυ2-4 [N308Q N407Q] mutant, however, a six-fold slower on-rate (8.3 x 10^4 $M^{-1}s^{-1}$) and a three-fold faster off-rate (6.2 x 10^{-3} s^{-1}) were observed. In studies of the kinetics of differentially glycosylated IgE-Fc fragments for FcεRI (either expressed on the surface of CHO cells or immobilised on an SPR chip), similar results were obtained: a recombinant IgE-Fc homologous to the IgY-Fc [N308Q] mutant, Cε2-4 [N265Q N371Q], was found to have only slightly lower affinity than a wild-type fragment in a cell binding assay, 5.0 x 10^{10} M^{-1} compared to 8.1 x 10^{10} M^{-1} (Young *et al.*, 1995). Although deglycosylation of IgG-Fc caused it to lose the ability to bind to receptors (Radaev and Sun, 2001), treatment of an IgE-Fc Cε3-4 fragment with PNGase reduced the affinity constant by only four-fold in an SPR assay, mainly by increasing the off-rate (Hunt *et al.*, 2005).

A comparison of the binding kinetics of Cυ2-4 and Cυ3-4 fragments was made in order to assess the involvement of the Cυ2 domains, which were suspected to be important due to the influence of the homologous Cε2 domains in stabilisation of the IgE high affinity receptor interaction (McDonnell *et al.*, 2001). As the recombinant IgY-Fc Cυ3-4 [wt] fragment was found to be somewhat artefactual (see chapter 5), the fragment most representative of an IgY-Fc with the Cυ2 domains removed is arguably the Cυ3-4 [C340S] mutant.

This mutant lacks the interchain disulfide located in Cυ2, but remains a covalently linked dimer by way of a disulfide between cysteines at position 347. Compared to Cυ2-4 [wt], this fragment has a roughly two-fold slower on-rate (2.2×10^5 $M^{-1}s^{-1}$) and roughly two-fold faster off-rate (5.2×10^5 s^{-1}). By contrast, compared to a Cε2-4 IgE-Fc, a Cε3-4 fragment has a three-fold faster on-rate and a five to ten-fold faster off-rate (Cook *et al.*, 1997; McDonnell *et al.*, 2001). These results appear to show that Cυ2 does not influence receptor binding kinetics to the same degree as Cε2 in IgE, although the lower on-rate could imply a role in maintenance of proper binding site orientation.

The presence and position of interchain disulfides were investigated using Cυ3-4 fragments. The Cυ3-4 [C347S] mutant, which has the interchain disulfide between a different, although ostensibly nearby, cysteine pair has a similar on-rate to the [C340S] mutant (2.0×10^5 $M^{-1}s^{-1}$), but a considerably faster off-rate (2.2×10^{-2} s^{-1}). A non-covalently linked Cυ3-4 mutant, [C340S C347S] was found to have a faster on-rate than either of the single disulfide Cυ3-4 mutants (4.1×10^5 $M^{-1}s^{-1}$), but a similar off-rate to the [C340S] mutant (7.4×10^{-3} s^{-1}). These results indicate that whilst interchain disulfides are not essential for Fc receptor engagement, the kinetics of Fc receptor binding are affected by either their location or absence. Reduced IgG and IgE are able to bind to cells expressing their high-affinity receptors, but with diminished affinity (McCool *et al.*, 1985; Takatsu *et al.*, 1975). In the case of IgE-Fc, a reduced Cε3-4 fragment was found to bind with 2:1 stoichiometry in solution (using AUC) and the affinity of a single binding sub-site (on a single Cε3 domain) could therefore be determined (Hunt *et al.*, 2005).

7.5 Discussion

In this chapter, the interaction between chicken serum IgY and Fc receptor(s) on a chicken monocyte cell line was investigated, with some preliminary characterisation of the influence of various structural features made using the recombinant IgY-Fc mutants designed and produced in the preceding chapters.

Specificity of the interaction was demonstrated by a lack of cross-reactivity between human and chicken antibodies and Fc receptors. This phenomenon has been previously noted at an effector response level and is one of the advantages of IgY technology in various immunological applications (Carlander *et al.*, 1999). Consistent with the assertion that the specificity is due to primary structure diversity between IgY and its mammalian homologues, rabbit polyclonal anti-human IgE was found to be unreactive with IgY adsorbed onto nitrocellulose (not shown).

The effect of IgY glycosylation on Fc receptor binding potential was extensively studied using mutagenesis of N-linked glycosylation sites in the Fc region or by enzymatic deglycosylation. At least one Fc glycosylation site is well conserved between IgY, IgG and IgE. As discussed in chapter 5 (sections 5.4.3 and 5.4.5.2), glycosylation appears to influence IgG and IgE structure in different ways, which has important functional consequences. Although glycosylation at asparagine 394 in Cε3 appears to be a requirement for production of a functional IgE-Fc in mammalian cells (Nettleton and Kochan, 1995), material (a) refolded from *E.coli*, which does not add glycosylation, and (b) expressed as a glycoprotein in mammalian cells and then enzymatically deglycosylated, both retain high affinity binding (Basu *et al.*, 1993; Geha *et al.*,

1985; Helm *et al.*, 1988; Hunt *et al.*, 2005). The Fc receptor binding potential of IgG and its Fc fragment, however, is effectively abrogated by deglycosylation with PNGase (Radaev and Sun, 2001), as shown as a control in figure 7.3.1 i. Similarly, Fc receptor recognition is completely lacking in aglycosylated forms produced in *E.coli* (Nose *et al.*, 1990) or in mammalian cells by mutagenesis of the homologous Cγ2 glycosylation site, asparagine 297 (Lund *et al.*, 2000; Tao and Morrison, 1989), or by inhibition of cellular glycosylation machinery using tunicamycin (Lund *et al.*, 1990; Walker *et al.*, 1989).

The influence of glycosylation on Fc receptor recognition in IgY therefore appears to be more like that of IgE than IgG. The pattern of glycosylation in IgY-Fc is most similar to that of IgE-Fc in terms of location and type of attached carbohydrate (Suzuki and Lee, 2004), so it might be expected that their sugars play similar roles. It is therefore somewhat surprising that aglycosylated IgY-Fc can be expressed by mammalian cells with the capacity to bind to monocyte Fc receptor, whereas IgE-Fc cannot – in order to retain receptor binding potential IgE-Fc fragments must be expressed with core glycosylation (Nettleton and Kochan, 1995) or subjected to refolding procedures (Geha *et al.*, 1985; Kenten *et al.*, 1984).

The effect of deglycosylation on receptor binding in IgG is the result of conformational changes which alter the orientation of the Cγ2 domains, making the horseshoe-like structure of the Fc more compact and the binding site sterically difficult to access (Krapp *et al.*, 2003; Mimura *et al.*, 2001). The structure of the Fc region in IgE, with its shorter high-mannose core oligosaccharide is already more 'closed' by comparison (Wan *et al.*, 2002; Wurzburg *et al.*, 2000), so its removal may have relatively minor conformational

consequences (Figure 7.5). Non-core glycosylation was found to have little effect on IgY-Fc receptor binding, whereas aglycosylated IgY-Fc had diminished binding affinity compared to native, by the same order of magnitude as deglycosylation of IgE-Fc. The similarity in the functional consequences when core oligosaccharides are removed from IgE-Fc and IgY-Fc suggests that the Fc region in IgY has a compact quaternary structure closer to that of IgE than IgG.

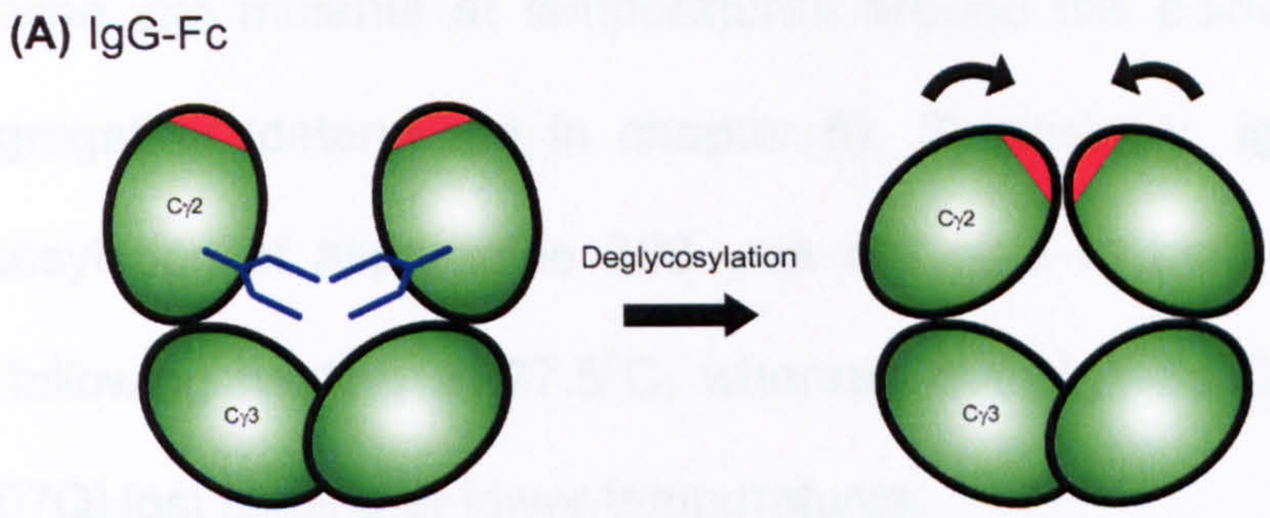


Figure 7.5
Structural changes in IgG-Fc and IgE-Fc induced by deglycosylation

Schematic showing alterations in quaternary structure of IgG-Fc (A) and IgE-Fc Cε3-4 (B) upon removal of core oligosaccharide. Crystal structures of IgG-Fc glycoforms with successively truncated oligosaccharide chains showed a gradual 'closure' of the Cγ2 domains (Krapp *et al.*, 2003). Removal of core oligosaccharide results in severely reduced receptor binding site accessibility (the region containing the binding site is shown in red). Compared to IgG-Fc, IgE-Fc has a shorter core oligosaccharide and adopts a more compact structure (Wurzburg *et al.*, 2000). As IgE-Fc is therefore already 'closed', deglycosylation causes a smaller conformational shift and receptor binding is only marginally affected.

Some unusual observations were made upon heating of IgY and the IgY-Fc glycosylation site mutants at temperatures around the point of thermally-induced aggregation (determined in chapter 5). Surprisingly, IgY-Fc (Cυ2-4) lacking glycosylation of asparagine 308 was still able to bind to MQ-NCSU monocytes following heating at 77.5°C, whereas both IgY-Fc Cυ2-4 [wt] and [N308Q N407Q] lost binding at lower temperatures.

These results seem inconsistent with the relationship between glycosylation state and melting temperature observed using DLS in chapter 5. However, DLS is unable to provide any indication of minor structural changes that do not result in aggregation events, therefore alterations could occur that precede complete unfolding. If structural rearrangements occur in the vicinity of the Cυ2 glycosylation site, this could explain the observed functional consequences. Depending on where the receptor binding site lies in IgY-Fc, it might be possible that N308-attached complex carbohydrate adopts a conformation after heating that sterically hinders receptor binding. This phenomenon could, however, be an artefact limited to the Fc fragment, as whole IgY did not behave in the same manner as Cυ2-4 [wt].

The presence of Cυ2 domains in IgY-Fc was unnecessary for the expression of a functional Cυ3-4 fragment and the cell binding kinetics were comparable, with only minor differences in the rates of association and dissociation. This shows that the major functional consequences observed when the homologous Cε2 domains are absent from IgE-Fc fragments were not found in IgY-Fc. However, a precise comparison of the influence of Cυ2 and Cε2 is difficult as IgY has a lower affinity than human IgE. The Cε2 domains are thought to contribute to the slow dissociation of IgE from its high affinity

receptor, FcεRI (McDonnell *et al.*, 2001). A conformational change in IgE upon receptor engagement has been postulated, in which the Cε2 domains, which are bent back to lie almost parallel with one of the Cε3 domains in the uncomplexed Fc (Wan *et al.*, 2002), swing out in order to form additional contacts with the receptor. In addition to suggestive crystallographic data, one of the strongest pieces of evidence for the involvement of Cε2 in the kinetics of IgE binding is the finding that Cε3-4 fragments dissociate ten times faster from FcεRIα than Cε2-4 fragments (Cook *et al.*, 1997; McDonnell *et al.*, 2001), although not all reports show such a dramatic difference (Henry *et al.*, 1997). It cannot be concluded that a conformational change in IgY-Fc does not occur upon receptor binding, although it would appear that Cυ2 is not as crucial a determinant of binding kinetics for IgY-Fc as is the homologous Cε2 for IgE-Fc.

The Cυ2 domains may instead play a more important role in determining correct folding (see chapter 5) and orientation of the putative binding domains, Cυ3. A four-fold increase in the off-rate of a Cυ3-4 fragment was observed when an interchain disulfide bond was introduced between cysteines at position 340, compared to an identical fragment with the cysteines at 347 instead. If the interchain disulfides in Cυ2 are crossed as they are in Cε2 (Wan *et al.*, 2002), then the C340 disulfide included in the engineered Cυ3-4 fragment would be artefactual, whereas the C347 disulfide (which does not have a homologue in IgE) would not. The observed effects of the C340 disulfide bridge could therefore be due to artefact-associated problems such as misfolding of Cυ3 in the region of the binding site or deleterious alteration of Cυ3 orientation, both of which are prevented by the presence of Cυ2.

A Cυ3-4 fragment lacking any covalent interchain bonds, [C340S C347S], was found to have slightly faster on- and off-rates compared to Cυ3-4 [C340S]. An *E.coli* produced IgE-Fc Cε3-4 fragment lacking an interchain disulfide (Cε3-4 ΔC) also has faster on- and off-rates compared to a deglycosylated disulfide-linked Cε3-4 produced in NS-0 cells, although considerably more so (Hunt *et al.*, 2005). Interestingly, the reported affinity of Cε3-4 ΔC for FcεRIα (4.14×10^7 M^{-1}) is similar to the affinity of Cυ3-4 [C340S C347S] for chicken monocytes (5.6×10^7 M^{-1}), although the assays used were different. For Cε3-4 ΔC, this was postulated to represent the affinity of a single binding site. If this is also the case for Cυ3-4 [C340S C347S], then the affinity of the individual binding sites may be remarkably similar in IgY-Fc and IgE-Fc.

The difference between the magnitude of the effect of disulfide removal in IgY-Fc and IgE-Fc may be due to structural differences. Gel filtration of Cε3-4 and Cε3-4 ΔC showed that the non-disulfide linked fragment eluted more slowly, despite identical migration on an SDS-PAGE gel, indicative of a more compact native structure (J. Hunt, 2004, PhD thesis). A mechanism in which disulfide linkage prevents the Cε3 domains 'rolling' past one another to adopt a more compact structure was envisioned. By contrast, the Cυ3-4 mutants were found to have almost identical gel filtration profiles (see chapter 5), which suggests that gross conformational differences do not occur in IgY-Fc when interchain disulfide bridges are removed. The influence of inter-heavy chain disulfide on IgY binding may be more similar to their influence in IgG; reduction of human IgG1 only lowers its capacity to activate U937 monocytes by ~20% (Lund *et al.*, 2000). As the inter-heavy chain disulfides in IgG occur in the disordered hinge region and Fc dimerisation occurs independently of them

(through non-covalent interactions between Cγ3 domains), it is perhaps not unexpected that the Cγ2 domains are largely unaffected by their absence. Although IgY does not contain a hinge region, the mode of binding could therefore be similar to that of IgG.

The next steps in these cell binding studies will be (a) to perform more replicates in order to increase the statistical significance of the observed differences, (b) to use a variety of methods to assess the binding parameters (for example, competition assays could be used to determine affinity constants in order to verify the values calculated from on- and off-rates) and (c) to take further measures to reduce errors; the two major limitations of the reported cell binding data are (i) the proportion of radioligand that has been inactivated by ^{125}I incorporation ('bindability') and (ii) the proportion of binding that is due to non-specific interactions – i.e. binding of radioligand to cells other than through their Fc receptors.

The majority of studies of this kind do not make adequate provision for the bindability of labelled proteins (Kermode, 1988). The labelling process can reduce the number of receptor-reactive species, so that the ligand concentration is over-estimated. Whilst this ought to have no effect on dissociation experiments, which are concentration independent, an accurate measure of the free ligand is crucial for determination of the rate of association. As receptor-reactivity ('bindability') was not determined, the dissociation data should therefore be considered the more reliable. Quality of radioligand is thought to be dependent upon the batch of ^{125}I used for labelling (Melson *et al.*, 1987). As all ^{125}I-labelled proteins used in this investigation were prepared at the same time, using the same batch of ^{125}I and used within a week, source decay and

bindability should be similar. Bindability can be determined by incubation of a fixed concentration of radioligand with increasing concentrations of receptor (i.e. receptor-bearing cells) to near excess. The percentage of reactive species is defined as the reciprocal of the extrapolated infinite receptor concentration (Kermode, 1988). Alternatively, if another system for ligand detection is available, equivalence between native and radiolabelled material can be measured, assuming native ligand has 100% bindability.

Non-specific binding was compensated for by subtraction of the quantity of radioligand bound to cells pre-blocked with unlabelled ligand following incubation over the timescale of each experiment. However, this method does not account for non-specific binding sites which have been blocked by unlabelled ligand, and it assumes that the proportion of non-specific to specific binding is constant, which is not necessarily the case. A reduction in error could be achieved using an improved method of compensation, although as non-specific binding was determined to be at most 10-15% of the total, the results presented would not be significantly different.

Chapter 8

Final discussion

8.1 Summary of reported findings

The overall aim of the work presented in this thesis was to perform some preliminary investigations into the structure and function of avian IgY, due to its phylogenetic relationship to both mammalian IgG and IgE. The preparation of homogeneous IgY-Fc fragments was highlighted as a crucial first step towards characterisation of the invariant portion of the antibody. IgY purified from chicken egg yolk was used to test the efficacy of papain digestion, which has historically been used to produce IgG-Fc for many structural and functional studies, including X-ray crystallographic determination of the Fc structure to high resolution (Deisenhofer, 1981). The Fc fragments yielded using this method comprised Cυ3-4, but were found to lack the Cυ2 domains. Crystals grown from papain-derived IgY-Fc were found to diffract X-rays, but to a resolution unsuitable for structural analysis. This was ascribed to protein heterogeneity resulting from an ill-defined proteolytic cleavage site.

To solve this problem, a recombinant 'full-length' IgY-Fc was engineered consisting of all three domains of the upsilon heavy chain that make up the Fc region, Cυ2-4. This approach had the added advantage that mutations could be made in order to increase homogeneity (with respect to glycosylation) and to study various structural elements within the Fc region. A mammalian expression system was developed so that large quantities of high-quality IgY-Fc

could be secreted into culture supernatants, from which purification was possible using affinity chromatography. Hits were obtained in crystallisation screens of full length IgY-Fc and efforts to optimise their growth and cryopreservation for use in X-ray diffraction studies are now underway.

The presence of IgY-Fc receptors analogous to Fc receptors for IgG and IgE were demonstrated on two important types of chicken leukocyte. Although these receptors could not be isolated as protein during the time of this investigation, two novel sequences homologous to Fc receptor subunits in mammals were cloned from chicken monocytes. Efforts were made to stably express the sequences in order to assay IgY-Fc binding, which will be completed in future experiments.

The combination of a receptor-expressing cell line and the production of mutant IgY-Fc fragments allowed several structural and functional observations to be made, in addition to the important step of confirming the functionality of the recombinant proteins. The finding that an IgY-Fc fragment consisting of Cυ3-4 was able to retain a comparable affinity for chicken monocytes as 'complete' IgY-Fc (Cυ2-4) suggests that Cυ2 is not involved IgY-Fc receptor binding. However, a IgE-Fc Cε3-4 fragment has also been shown to bind to FcεRI with a similar affinity to a Cε2-4 fragment (Cook *et al.*, 1997), although with markedly different kinetics (McDonnell *et al.*, 2001).

IgY, like IgG and IgE, is a heterotetrameric glycoprotein joined by covalent disulfide bonds. The number of heavy chain constant domains, the pattern and type of N-linked glycosylation and the location of putative inter-heavy chain disulfide bonds has been suggested to be more similar to that of IgE than IgG (Parvari *et al.*, 1988; Suzuki and Lee, 2004), which could indicate

that their structures are similar. However, the affinity and kinetics of IgY-Fc receptor binding, as reported in this thesis, were found to be closer to that of IgG, which makes determination of the structure of IgY highly informative. The influence of several structural features was examined in comparison with similar experiments previously reported for both IgG and IgE.

IgY-Fc was found to retain the capacity to bind to its monocyte receptor despite removal of carbohydrate using a deglycosylating enzyme, or mutagenesis of glycosylation sites to produce an aglycosylated form. By contrast, similar experiments with mammalian IgG demonstrated that receptor binding is completely abrogated (Lund *et al.*, 1990; Radaev and Sun, 2001; Tao and Morrison, 1989). Radiolabelled, aglycosylated IgY-Fc was found to bind to chicken monocytes with more than ten fold lower affinity than IgY-Fc containing two glycosylation sites per chain, primarily due to a significantly faster on-rate. Although a lesser effect is seen when IgE-Fc is deglycosylated (Hunt *et al.*, 2005), the structural contribution of glycosylation appears to be similar in IgY and IgE.

A series of dynamic light scattering (DLS) experiments determined that the thermal stability of IgY was more IgG-like; the approximate melting temperature of IgY was found to lie between that of human and rabbit IgG, whereas IgE has been shown to be significantly more susceptible to heat inactivation. This was further confirmed by comparison Cε2-4 and Cυ2-4 fragments using the same technique. Glycosylation of the IgY-Fc region was shown to be important in maintenance of Fc stability and solubility.

The sequence of the upsilon heavy chain suggests that IgY may possess one additional inter-heavy chain disulfide bond than IgE, which was supported

by the finding that no free sulfhydryls were detected using an Ellman assay. A series of Cυ3-4 fragments were used to examine the consequences of removal of inter-heavy chain disulfides on receptor recognition. Surprisingly, inclusion of C340 and C347 in the same Cυ3-4 fragment caused the production of IgY-Fc fragments lacking inter-heavy chain disulfide bonds (i.e. an intra-chain bond may have formed instead). This was not the case in fragments containing Cυ2 domains or when one or the other of these cysteine residues was mutated to serine. A Cυ3-4 fragment with a single inter-heavy chain disulfide bond between cysteines at position 340 had a significantly faster rate of dissociation from chicken monocytes compared to a similar fragment with the disulfide bond between cysteines at position 347 instead. Taken together, these results suggest that the disulfide bond between C340 forms in an artefactual manner in IgY-Fc fragments lacking complete Cυ2 domains. In the complete Fc, the cysteine at position 340 may form a crossed inter-heavy chain disulfide bond with cysteine 252 in the opposite chain, not with C340, which would be homologous to C241 and C328 in IgE-Fc, as revealed by the crystal structure of a Cε2-4 fragment (Wan *et al.*, 2002). Whilst the artefactual formation of a C340-C340 parallel disulfide bond does not entirely abrogate binding to cells, the affinity is somewhat lowered. Given that the Fc receptor binding sites in IgG and IgE reside in this region (the CH3 N-terminus), this result might imply that the receptor binding site maps to a similar region in IgY-Fc.

A Cυ3-4 fragment lacking inter-heavy chain disulfides produced by mutation of both cysteine 340 and cysteine 347 to serine was found to have comparable affinity to Cυ3-4 linked by a single inter-heavy chain bond. Experiments reported in the literature with IgE-Fc and IgG-Fc, albeit using

different methodology, suggest that disulfide linkage is more important in maintenance of receptor binding kinetics in IgE than IgG (Hunt *et al.*, 2005; Lund *et al.*, 2000), which would make IgY more like IgG in this regard. Furthermore, removal of inter-heavy chain disulfide bonds made little or no difference to the gel filtration behaviour of Cυ3-4 fragments, whereas a significant shift in gel retention time was observed when an IgE-Fc Cε3-4 fragment was compared to a similar fragment lacking inter-heavy chain disulfide bonds (J. Hunt, 2004, PhD Thesis), which was interpreted as a change in Cε3 domain orientation and overall Fc shape. For IgY, this suggests that removal of inter-heavy chain disulfides has less of an impact upon IgY-Fc structure and/or the mode of Fc receptor binding is different to the IgE:FcεRI interaction and more like that of an IgG:FcγR.

The function of Cυ2 in IgY-Fc was an interesting question to address given the importance of the equivalent domain in IgE, Cε2, in determining the kinetics of the interaction with FcεRI. Although a difference in the cell binding kinetics was noted between a Cυ2-4 fragment (wt) and a Cυ3-4 fragment (the C340S mutant, which is probably the best representation of a Cυ2-deleted IgY-Fc), the change in the rate of dissociation when Cυ2 is absent (~2 fold faster) was relatively minor compared to the ~10 fold or greater increase in dissociation rate when the binding of Cε3-4 fragments is compared to that of the complete IgE-Fc (Cε2-4) using surface plasmon resonance (Cook *et al.*, 1997; McDonnell *et al.*, 2001). Thus Cυ2 may not play the same role in IgY as Cε2 does in IgE.

8.2 Insights into the evolution of IgG and IgE

One of the reasons for studying IgY was to help to elucidate further the various structural and functional developments specific to IgG and IgE in order to gain a better appreciation of how these isotypes evolved such disparate roles in mammals. The work presented in this thesis provides crucial materials and a useful framework for the design of future experiments to address this aim. Several key differences between IgY and IgE revealed by this investigation are worthy of further study.

IgE has been known to be substantially more heat labile than IgG for many years, with instability mapping to the Fc region (Dorrington and Bennich, 1978). Recently, experiments using ANS dye fluorimetry and NMR spectroscopy have suggested that this may be the result of the Cε3 domains existing in a partially unfolded 'molten globule' state, with receptor (FcεRI) engagement required for complete folding (Harwood *et al.*, 2006; Price *et al.*, 2005). This may allow the IgE-Fc:FcεRI interaction to be fine-tuned in order to increase specificity and affinity (Harwood and McDonnell, 2007). Although identical experiments have yet to be performed using the homologous Cυ3 domains in IgY, the melting temperature of the IgY-Fc region was found to be significantly higher than that of the IgE-Fc region, which would suggest that Cυ3 is not as thermally unstable as Cε3 and may not be a molten globule. The 'molten globule' state of Cε3 could therefore represent one of the major adaptations of IgE-Fc to a high affinity binding mode.

A number of results reported in this thesis imply similarities between the structure of IgY-Fc and IgE-Fc; work is underway to use the recombinant

functional Cυ2-4 fragments prepared in this investigation to solve the crystal structure of the complete Fc region of IgY. Cell binding experiments reported in chapter 7 show that the absence of Cυ2 from an IgY-Fc fragment did not have the same degree of functional consequence as has been demonstrated when Cε2 domains are absent from an IgE-Fc fragment. One of the most striking features of IgE compared to IgG is the acute bend and asymmetry of the Fc region. A change in this structure upon IgE-Fc binding to receptor is believed to play an important role in determining the receptor binding kinetics. A detailed analysis of the structural differences between Cυ2 and Cε2 in the context of complete Fc fragments, in order to discover the precise cause of their functional differences, could therefore be highly informative.

The kinetics of IgY-Fc binding to monocyte Fc receptor was shown to be dependent on the number and location of inter-heavy chain disulfide bonds. Although the locations of most disulfide bonds in IgY-Fc are conserved in IgE-Fc, there are differences; a third inter-heavy chain bond is present in IgY-Fc in a location equivalent to the binding site for FcεRI in IgE-Fc. Subtle alterations in the pattern of disulfide bonds during the divergence of IgG and IgE may have had important consequences for their Fc receptor interactions.

Similarly, the pattern of glycosylation was found to play an important role in the maintenance of both IgY-Fc structure and function, just as in both IgE-Fc and IgG-Fc. In IgG-Fc, however, a modification of the type of core oligosaccharide has been made, i.e. complex-type rather than high mannose, with significant consequences for the quaternary structure of the Ig domains. The composition of attached carbohydrates has recently been proposed as another level of control over the nature of effector responses induced by

antibody-dependent mechanisms (Davies *et al.*, 2001; Matsumiya *et al.*, 2007; Shields *et al.*, 2002; Shinkawa *et al.*, 2003; Umana *et al.*, 1999).

8.3 Addendum: new developments

Two new developments have occurred too close to the date of submission of this thesis to have been included elsewhere. Firstly, as a continuation of the results reported in chapter 6, a cell line stably expressing both human FcεRIα and the chicken homologue of FcRγ has been created. The residues in human FcRγ that interact with human FcεRIα to allow subunit association, which is required for FcεRIα surface expression (Wines *et al.*, 2006), are conserved in the chicken homologue of FcRγ. FcεRIα was detected both internally and externally, which suggests that chimeric chicken/human receptor complexes have been formed (Figure 8.3). These cells will be used to confirm the signalling potential of the putative chicken FcRγ orthologue.

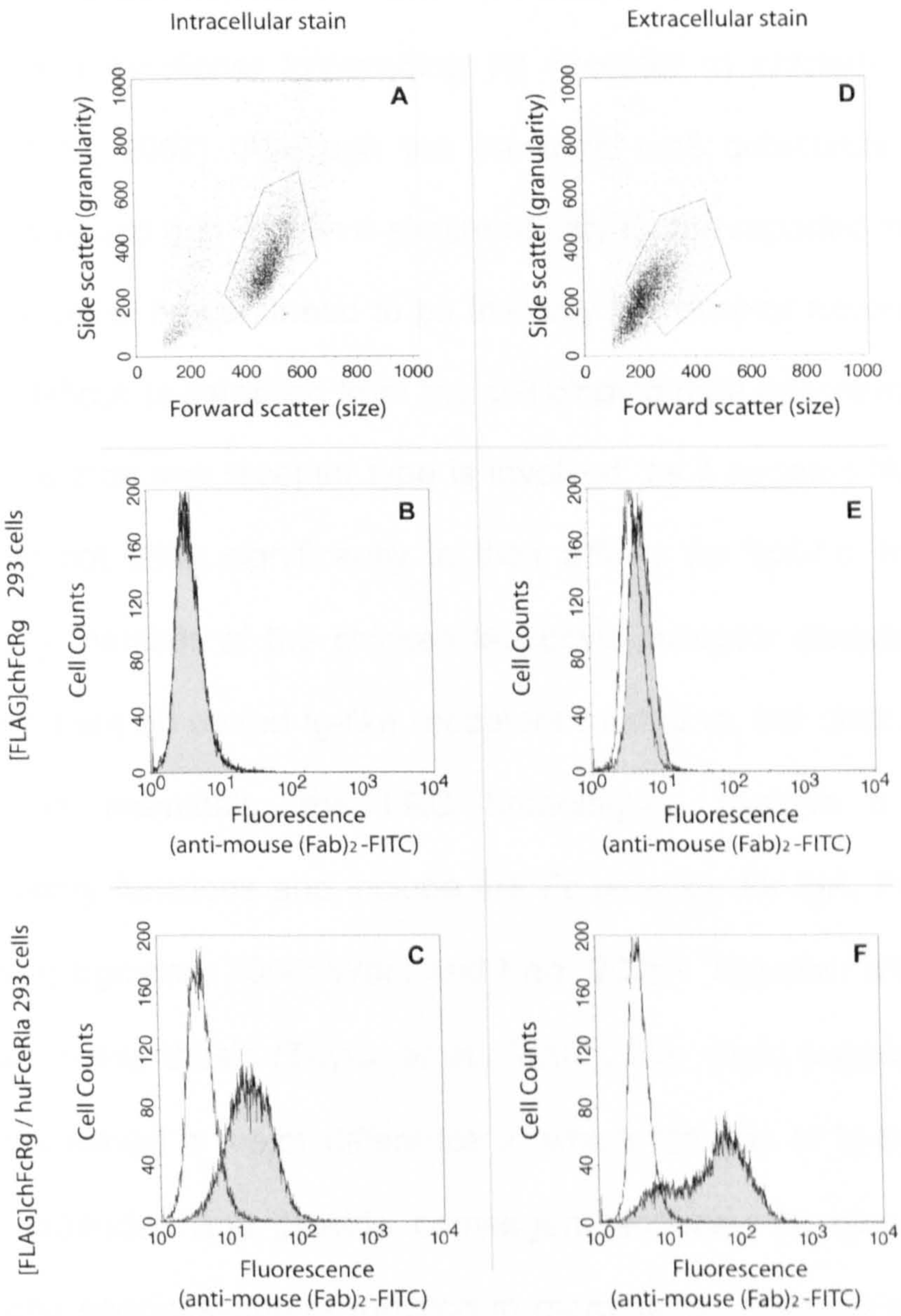


Figure 8.3
Chicken FcRγ can substitute for human FcRγ in a chimeric FcεRI complex

FACS profiles showing HEK 293 cells gated to exclude dead cells (A & D) following incubation with 1μg/ml mouse monoclonal anti-FcεRIα (15.1) antibodies followed by 10μg/ml anti-mouse-FITC (filled peaks) or with buffer alone followed by 10μg/ml anti-mouse-FITC (unfilled peaks). Cells were fixed, washed and incubated in the presence (A, B & C) or absence (D, E & F) of 0.1% saponin for intracellular or extracellular staining respectively. The staining pattern of cells stably transfected with both the vector for the [FLAG]chFcRγ construct and a pCDNA3-based vector encoding human FcεRIα (C & F) are shown in comparison with cells transfected with the vector for the [FLAG]chFcRγ construct alone (B & E).

The second development is that Viertlboeck *et al.* have reported the identification of a functional IgY-binding Fc receptor in chicken, CHIR-AB1 (Viertlboeck *et al.*, 2007). Although the transcript was detectable in chicken monocytes and bound IgY-Fc with a similar affinity to that reported in chapter 7, CHIR-AB1 has yet to be confirmed to be the only Fc receptor expressed in this cell type. It is difficult to establish from the cell binding data described in chapter 7 whether more than one receptor type is involved, as it appears likely that the receptors may not differ significantly in their affinity for IgY-Fc. Interestingly, CHIR-AB1 is a member of the chicken leukocyte receptor complex (LRC), a family of more than 60 paired Ig-like receptors related to, but distinct from, the FcR family. In mammals, the LRC homologues perform a variety of immunoregulatory functions and include the Fc receptor for IgA, FcαR / CD89 (Otten and van Egmond, 2004; Woof and Kerr, 2006). Together with published data reported in this thesis (Taylor *et al.*, 2007), this could suggest that birds and mammals exhibit a major difference in which families of Ig-like receptors have been expanded and provide convergent antibody Fc recognition. The separation and specialisation of function in mammalian antibody isotypes may therefore have evolved not only through structural alterations in the antibodies themselves, but also through changes in their receptors.

References

Allen, J. M., and Seed, B. (1989). Isolation and expression of functional high-affinity Fc receptor complementary DNAs. Science *243*, 378-381.

Ambrosius, H., and Hadge, D. (1987). Chicken immunoglobulins. Vet Immunol Immunopathol *17*, 57-67.

Amemiya, C. T., Haire, R. N., and Litman, G. W. (1989). Nucleotide sequence of a cDNA encoding a third distinct Xenopus immunoglobulin heavy chain isotype. Nucleic Acids Res *17*, 5388.

Amzel, L. M., and Poljak, R. J. (1979). Three-dimensional structure of immunoglobulins. Annu Rev Biochem *48*, 961-997.

Anderson, C. L., and Abraham, G. N. (1980). Characterization of the Fc receptor for IgG on a human macrophage cell line, U937. J Immunol *125*, 2735-2741.

Asara, J. M., Schweitzer, M. H., Freimark, L. M., Phillips, M., and Cantley, L. C. (2007). Protein sequences from mastodon and Tyrannosaurus rex revealed by mass spectrometry. Science *316*, 280-285.

Aschaffenburg, R., Lewis, M., Phillips, D. C., Press, E. M., Smith, S. G., Sutton, B. J., and Mountford, C. W. (1979). Crystallographic studies of immunoglobulins: crystallization of the Fc fragment of rabbit IgG with and without cleavage of the inter-chain disulphide bridge. J Mol Biol *135*, 1033-1036.

Atwell, J. L., and Marchalonis, J. J. (1977). Immunoglobulin gamma chains of a monotreme mammal, the echidna (Tachyglossus aculeatus): amino acid composition and partial amino acid sequence. J Immunogenet *4*, 73-80.

Avery, S., Rothwell, L., Degen, W. D., Schijns, V. E., Young, J., Kaufman, J., and Kaiser, P. (2004). Characterization of the first nonmammalian T2 cytokine gene cluster: the cluster contains functional single-copy genes for IL-3, IL-4, IL-13, and GM-CSF, a gene for IL-5 that appears to be a pseudogene, and a gene encoding another cytokinelike transcript, KK34. J Interferon Cytokine Res *24*, 600-610.

Baenziger, J., Kornfeld, S., and Kochwa, S. (1974a). Structure of the carbohydrate units of IgE immunoglobulin. I. Over-all composition, glycopeptide isolation, and structure of the high mannose oligosaccharide unit. J Biol Chem *249*, 1889-1896.

Baenziger, J., Kornfeld, S., and Kochwa, S. (1974b). Structure of the carbohydrate units of IgE immunoglobulin. II. Sequence of the sialic acid-containing glycopeptides. J Biol Chem *249*, 1897-1903.

Barkas, T., and Watson, C. M. (1979). Induction of an Fc conformational change by binding of antigen: the generation of protein A-reactive sites in chicken immunoglobulin. Immunology *36*, 557-561.

Basu, M., Hakimi, J., Dharm, E., Kondas, J. A., Tsien, W. H., Pilson, R. S., Lin, P., Gilfillan, A., Haring, P., Braswell, E. H., and *et al.* (1993). Purification and characterization of human recombinant IgE-Fc fragments that bind to the human high affinity IgE receptor. J Biol Chem *268*, 13118-13127.

Bebbington, C. R., Renner, G., Thomson, S., King, D., Abrams, D., and Yarranton, G. T. (1992). High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. Biotechnology (N Y) *10*, 169-175.

Bellavia, A., Marino, V., Gallo, E., Peri, S. M., Bentivegna, C., Agresti, L., and Di Bona, M. (1992). Contact sensitivity to oxazolone in the chicken: evidence for Arthus type hypersensitivity of the cutaneous reaction. Immunopharmacol Immunotoxicol *14*, 233-250.

Bendtsen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. J Mol Biol *340*, 783-795.

Bininda-Emonds, O. R., Cardillo, M., Jones, K. E., MacPhee, R. D., Beck, R. M., Grenyer, R., Price, S. A., Vos, R. A., Gittleman, J. L., and Purvis, A. (2007). The delayed rise of present-day mammals. Nature *446*, 507-512.

Bjerke, T., Nielsen, S., Helgestad, J., Nielsen, B. W., and Schiotz, P. O. (1993). Purification of human blood basophils by negative selection using immunomagnetic beads. J Immunol Methods *157*, 49-56.

Bjorklund, J. E., Karlsson, T., and Magnusson, C. G. (1999). N-glycosylation influences epitope expression and receptor binding structures in human IgE. Mol Immunol *36*, 213-221.

Bjorklund, J. E., Schmidt, M., and Magnusson, C. G. (2000). Characterisation of recombinant human IgE-Fc fragments expressed in baculovirus-infected insect cells. Mol Immunol *37*, 169-177.

Blank, U., Ra, C., Miller, L., White, K., Metzger, H., and Kinet, J. P. (1989). Complete structure and expression in transfected cells of high affinity IgE receptor. Nature *337*, 187-189.

Boguski, M. S., Lowe, T. M., and Tolstoshev, C. M. (1993). dbEST--database for "expressed sequence tags". Nat Genet *4*, 332-333.

Boss, M. A., Kenten, J. H., Wood, C. R., and Emtage, J. S. (1984). Assembly of functional antibodies from immunoglobulin heavy and light chains synthesised in E. coli. Nucleic Acids Res *12*, 3791-3806.

Breathnach, R., Benoist, C., O'Hare, K., Gannon, F., and Chambon, P. (1978). Ovalbumin gene: evidence for a leader sequence in mRNA and DNA sequences at the exon-intron boundaries. Proc Natl Acad Sci U S A *75*, 4853-4857.

Brown, J. W., Payne, R. B., and Mindell, D. P. (2007). Nuclear DNA does not reconcile 'rocks' and 'clocks' in Neoaves: a comment on Ericson *et al.* Biol Lett *3*, 257-259; discussion 260-251.

Burge, C., and Karlin, S. (1997). Prediction of complete gene structures in human genomic DNA. J Mol Biol *268*, 78-94.

Burt, D. W. (2005). Chicken genome: current status and future opportunities. Genome Res *15*, 1692-1698.

Bylund, D. B., and Toews, M. L. (1993). Radioligand binding methods: practical guide and tips. Am J Physiol *265*, L421-429.

Capron, A., Dombrowicz, D., and Capron, M. (2004). Helminth infections and allergic diseases: from the Th2 paradigm to regulatory networks. Clin Rev Allergy Immunol *26*, 25-34.

Carlander, D., Stalberg, J., and Larsson, A. (1999). Chicken antibodies: a clinical chemistry perspective. Ups J Med Sci *104*, 179-189.

Carlson, H. C., and Allen, J. R. (1969). The acute inflammatory reaction in chicken skin: blood cellular response. Avian Dis *13*, 817-833.

Catterall, J. F., O'Malley, B. W., Robertson, M. A., Staden, R., Tanaka, Y., and Brownlee, G. G. (1978). Nucleotide sequence homology at 12 intron--exon junctions in the chick ovalbumin gene. Nature *275*, 510-513.

Chand, N., Carlson, H. C., and Eyre, P. (1976). Passive cutaneous anaphylaxis in the domestic fowl. Int Arch Allergy Appl Immunol *51*, 508-517.

Chartrand, S. L., Litman, G. W., Lapointe, N., Good, R. A., and Frommel, D. (1971). The evolution of the immune response. XII. The immunoglobulins of the turtle. Molecular requirements for biologic activity of 5. J Immunol *107*, 1-11.

Coca, A. F., and Grove, E. F. (1925). Studies in Hypersensitiveness: XIII. A Study of the Atopic Reagins. J Immunol *10*, 445-464.

Cockett, M. I., Bebbington, C. R., and Yarranton, G. T. (1990). High level expression of tissue inhibitor of metalloproteinases in Chinese hamster ovary cells using glutamine synthetase gene amplification. Biotechnology (N Y) *8*, 662-667.

Cohen-Solal, J. F., Cassard, L., Fridman, W. H., and Sautes-Fridman, C. (2004). Fc gamma receptors. Immunol Lett 92, 199-205.

Conrad, D. H., Bazin, H., Sehon, A. H., and Froese, A. (1975). Binding parameters of the interaction between rat IgE and rat mast cell receptors. J Immunol *114*, 1688-1691.

Conrad, D. H., Berczi, I., and Froese, A. (1976). Characterization of the target cell receptor for IgE-I. Solubilization of IgE-receptor complexes from rat mast cells and rat basophilic leukemia cells. Immunochemistry *13*, 329-332.

Conrad, D. H., and Froese, A. (1978). Characterization of the target cell receptor for IgE--IV. Isolation of IgE-receptor complexes. Immunochemistry *15*, 283-288.

Conrad, D. H., Wingard, J. R., and Ishizaka, T. (1983). The interaction of human and rodent IgE with the human basophil IgE receptor. J Immunol *130*, 327-333.

Conroy, M. C., Adkinson, N. F., Jr., and lichtenstein, L. M. (1977). Measurement of IgE on human basophils: relation to serum IgE and anti-IgE-induced histamine release. J Immunol *118*, 1317-1321.

Cook, J. P., Henry, A. J., McDonnell, J. M., Owens, R. J., Sutton, B. J., and Gould, H. J. (1997). Identification of contact residues in the IgE binding site of human FcepsilonRIalpha. Biochemistry *36*, 15579-15588.

Cook, S. F. (1937). A study of blood picture of poultry and its diagnostic significance. Poultry Sci *16*, 291.

Cookson, E. J., and Beynon, R. J. (1987). Degradation artefacts during sample preparation for sodium dodecyl sulphate polyacrylamide gel electrophoresis. Biosci Rep 7, 209-215.

Cooper, M. D., and Alder, M. N. (2006). The evolution of adaptive immune systems. Cell *124*, 815-822.

Danilova, N., Bussmann, J., Jekosch, K., and Steiner, L. A. (2005). The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. Nat Immunol *6*, 295-302.

Davies, J., Jiang, L., Pan, L. Z., LaBarre, M. J., Anderson, D., and Reff, M. (2001). Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: Expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FC gamma RIII. Biotechnol Bioeng *74*, 288-294.

Davis, R. S., Dennis, G., Jr., Odom, M. R., Gibson, A. W., Kimberly, R. P., Burrows, P. D., and Cooper, M. D. (2002). Fc receptor homologs: newest members of a remarkably diverse Fc receptor gene family. Immunol Rev *190*, 123-136.

Davis, R. S., Ehrhardt, G. R., Leu, C. M., Hirano, M., and Cooper, M. D. (2005). An extended family of Fc receptor relatives. Eur J Immunol *35*, 674-680.

Davis, R. S., Wang, Y. H., Kubagawa, H., and Cooper, M. D. (2001). Identification of a family of Fc receptor homologs with preferential B cell expression. Proc Natl Acad Sci U S A *98*, 9772-9777.

Degen, W. G., Daal, N., Rothwell, L., Kaiser, P., and Schijns, V. E. (2005). Th1/Th2 polarization by viral and helminth infection in birds. Vet Microbiol *105*, 163-167.

Deisenhofer, J. (1981). Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8-A resolution. Biochemistry *20*, 2361-2370.

Doolittle, R. F., and Bork, P. (1993). Evolutionarily mobile modules in proteins. Sci Am *269*, 50-56.

Dorrington, K. J., and Bennich, H. (1973). Thermally induced structural changes in immunoglobulin E. J Biol Chem *248*, 8378-8384.

Dorrington, K. J., and Bennich, H. H. (1978). Structure-function relationships in human immunoglobulin E. Immunol Rev *41*, 3-25.

Dreesman, G., and Benedict, A. A. (1965a). Properties of papain-digested chicken 7 S gamma-globulin. J Immunol *95*, 855-866.

Dreesman, G. R., and Benedict, A. A. (1965b). Reductive dissociation of chicken gamma-G immunoglobulin in neutral solvents without a dispersing agent. Proc Natl Acad Sci U S A *54*, 822-830.

Ehrhardt, G. R., Davis, R. S., Hsu, J. T., Leu, C. M., Ehrhardt, A., and Cooper, M. D. (2003). The inhibitory potential of Fc receptor homolog 4 on memory B cells. Proc Natl Acad Sci U S A *100*, 13489-13494.

Eldin, P., Pauza, M. E., Hieda, Y., Lin, G., Murtaugh, M. P., Pentel, P. R., and Pennell, C. A. (1997). High-level secretion of two antibody single chain Fv fragments by Pichia pastoris. J Immunol Methods *201*, 67-75.

Ellman, G. L. (1958). A colorimetric method for determining low concentrations of mercaptans. Arch Biochem Biophys *74*, 443-450.

Ericson, P. G., Anderson, C. L., Britton, T., Elzanowski, A., Johansson, U. S., Kallersjo, M., Ohlson, J. I., Parsons, T. J., Zuccon, D., and Mayr, G. (2006). Diversification of Neoaves: integration of molecular sequence data and fossils. Biol Lett *2*, 543-547.

Ericson, P. G., Anderson, C. L., and Mayr, G. (2007). Reply. Hangin' on to our rocks 'n clocks: a reply to Brown *et al.* Biol Lett *3*, 260-261.

Ernst, L. K., Duchemin, A. M., and Anderson, C. L. (1993). Association of the high-affinity receptor for IgG (Fc gamma RI) with the gamma subunit of the IgE receptor. Proc Natl Acad Sci U S A *90*, 6023-6027.

Ernst, L. K., Duchemin, A. M., Miller, K. L., and Anderson, C. L. (1998). Molecular characterization of six variant Fcgamma receptor class I (CD64) transcripts. Mol Immunol *35*, 943-954.

Faith, R. E., and Clem, L. W. (1973). Passive cutaneous anaphylaxis in the chicken. Biological fractionation of the mediating antibody population. Immunology *25*, 151-164.

Fayngerts, S. A., Najakshin, A. M., and Taranin, A. V. (2007). Species-specific evolution of the FcR family in endothermic vertebrates. Immunogenetics *59*, 493-506.

Feinstein, A., and Munn, E. A. (1969). Conformation of the free and antigen-bound IgM antibody molecules. Nature *224*, 1307-1309.

Fellah, J. S., and Charlemagne, J. (1988). Characterization of an IgY-like low molecular weight immunoglobulin class in the Mexican axolotl. Mol Immunol *25*, 1377-1386.

Fellah, J. S., Kerfourn, F., Wiles, M. V., Schwager, J., and Charlemagne, J. (1993). Phylogeny of immunoglobulin heavy chain isotypes: structure of the constant region of Ambystoma mexicanum upsilon chain deduced from cDNA sequence. Immunogenetics *38*, 311-317.

Finkelman, F. D., Rothenberg, M. E., Brandt, E. B., Morris, S. C., and Strait, R. T. (2005). Molecular mechanisms of anaphylaxis: lessons from studies with murine models. J Allergy Clin Immunol *115*, 449-457; quiz 458.

Flajnik, M. F. (2002). Comparative analyses of immunoglobulin genes: surprises and portents. Nat Rev Immunol *2*, 688-698.

Flanagan, J. G., and Rabbitts, T. H. (1982). The sequence of a human immunoglobulin epsilon heavy chain constant region gene, and evidence for three non-allelic genes. Embo J *1*, 655-660.

Fleischman, J. B., Porter, R. R., and Press, E. M. (1963). The Arrangement of the Peptide Chains in Gamma-Globulin. Biochem J *88*, 220-228.

Froese, A. (1980). Structure and function of the receptor for IgE. Crit Rev Immunol *1*, 79-132.

Fujiki, K., Shin, D. H., Nakao, M., and Yano, T. (2000). Molecular cloning and expression analysis of carp (Cyprinus carpio) interleukin-1 beta, high affinity immunoglobulin E Fc receptor gamma subunit and serum amyloid A. Fish Shellfish Immunol *10*, 229-242.

Furlong, R. F. (2005). Insights into vertebrate evolution from the chicken genome sequence. Genome Biol *6*, 207.

Galfre, G., and Milstein, C. (1981). Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol *73*, 3-46.

Gall, W. E., Cunningham, B. A., Waxdal, M. J., Konigsberg, W. H., and Edelman, G. M. (1968). The covalent structure of a human gamma G-immunoglobulin. IV. The interchain disulfide bonds. Biochemistry 7, 1973-1982.

Garman, S. C., Wurzburg, B. A., Tarchevskaya, S. S., Kinet, J. P., and Jardetzky, T. S. (2000). Structure of the Fc fragment of human IgE bound to its high-affinity receptor Fc epsilonRI alpha. Nature *406*, 259-266.

Geha, R. S., Helm, B., and Gould, H. (1985). Inhibition of the Prausnitz-Kustner reaction by an immunoglobulin epsilon-chain fragment synthesized in E. coli. Nature *315*, 577-578.

Geuijen, C. A., Clijsters-van der Horst, M., Cox, F., Rood, P. M., Throsby, M., Jongeneelen, M. A., Backus, H. H., van Deventer, E., Kruisbeek, A. M., Goudsmit, J., and de Kruif, J. (2005). Affinity ranking of antibodies using flow cytometry: application in antibody phage display-based target discovery. J Immunol Methods *302*, 68-77.

Ghetie, V., and Ward, E. S. (2000). Multiple roles for the major histocompatibility complex class I- related receptor FcRn. Annu Rev Immunol *18*, 739-766.

Ghirlando, R., Lund, J., Goodall, M., and Jefferis, R. (1999). Glycosylation of human IgG-Fc: influences on structure revealed by differential scanning micro-calorimetry. Immunol Lett *68*, 47-52.

Gobel, T. W., and Bolliger, L. (1998). The chicken TCR zeta-chain restores the function of a mouse T cell hybridoma. J Immunol *160*, 1552-1554.

Goodrich, J. A., and Kugel, J. F. (2007). Binding and kinetics for molecular biologists (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press).

Gould, H., Sutton, B., Beavil, A., Edmeades, R., and Martin, D. (1991). Immunoglobulin E receptors. Clin Exp Allergy *21 Suppl 1*, 138-147.

Gould, H. J., Sutton, B. J., Beavil, A. J., Beavil, R. L., McCloskey, N., Coker, H. A., Fear, D., and Smurthwaite, L. (2003). The biology of IGE and the basis of allergic disease. Annu Rev Immunol *21*, 579-628.

Gounni, A. S., Wellemans, V., Yang, J., Bellesort, F., Kassiri, K., Gangloff, S., Guenounou, M., Halayko, A. J., Hamid, Q., and Lamkhioued, B. (2005). Human airway smooth muscle cells express the high affinity receptor for IgE (Fc epsilon RI): a critical role of Fc epsilon RI in human airway smooth muscle cell function. J Immunol *175*, 2613-2621.

Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol *36*, 59-74.

Grey, H. M. (1967a). Duck immunoglobulins. I. Structural studies on a 5.7S and 7.8S gamma-globulin. J Immunol *98*, 811-819.

Grey, H. M. (1967b). Duck immunoglobulins. II. Biologic and immunochemical studies. J Immunol *98*, 820-826.

Guselnikov, S. V., Bell, A., Najakshin, A. M., Robert, J., and Taranin, A. V. (2003). Signaling FcRgamma and TCRzeta subunit homologs in the amphibian Xenopus laevis. Dev Comp Immunol *27*, 727-733.

Guselnikov, S. V., Erilova, A., Najakshin, A.M., Cohen, N., Robert, J. and Taranin, A. V. (2004). FcR-like genes in the amphibian Xenopus: species-specific expansion, structural diversity, and developmental expression., Paper presented at: 12th Int. Cong. Immunol. and 4th Annu. Conf. FOCIS (Montreal).

Haba, S., and Nisonoff, A. (1991). Proteolytic digestion of mouse IgE. J Immunol Methods *138*, 15-23.

Haeffner-Cavaillon, N., Dorrington, K. J., and Klein, M. (1979a). Studies on the Fc gamma receptor of the murine macrophage-like cell line P388D1. II. Binding of human IgG subclass proteins and their proteolytic fragments. J Immunol *123*, 1914-1919.

Haeffner-Cavaillon, N., Klein, M., and Dorrington, K. J. (1979b). Studies on the Fc gamma receptor of the murine macrophage-like cell line P388D1. I. The binding of homologous and heterologous immunoglobulin G1. J Immunol *123*, 1905-1913.

Haisch, K., Gibbs, B. F., Korber, H., Ernst, M., Grage-Griebenow, E., Schlaak, M., and Haas, H. (1999). Purification of morphologically and functionally intact human basophils to near homogeneity. J Immunol Methods *226*, 129-137.

Hansen, J. D., Landis, E. D., and Phillips, R. B. (2005). Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. Proc Natl Acad Sci U S A *102*, 6919-6924.

Harris, L. J., Larson, S. B., and McPherson, A. (1999). Comparison of intact antibody structures and the implications for effector function. Adv Immunol *72*, 191-208.

Harris, L. J., Skaletsky, E., and McPherson, A. (1998). Crystallographic structure of an intact IgG1 monoclonal antibody. J Mol Biol *275*, 861-872.

Harwood, N. E., and McDonnell, J. M. (2007). The intrinsic flexibility of IgE and its role in binding FcepsilonRI. Biomed Pharmacother *61*, 61-67.

Harwood, N. E., Price, N. C., and McDonnell, J. M. (2006). Catalytic folding of the Cepsilon3 domain by its high affinity receptor. FEBS Lett *580*, 2129-2134.

Hatta, H., Tsuda, K., Akachi, S., Kim, M., and Yamamoto, T. (1993). Productivity and some properties of egg yolk antibody (IgY) against human rotavirus compared with rabbit IgG. Biosci Biotechnol Biochem *57*, 450-454.

Hatzivassiliou, G., Miller, I., Takizawa, J., Palanisamy, N., Rao, P. H., Iida, S., Tagawa, S., Taniwaki, M., Russo, J., Neri, A. *et al.* (2001). IRTA1 and IRTA2, novel immunoglobulin superfamily receptors expressed in B cells and involved in chromosome 1q21 abnormalities in B cell malignancy. Immunity *14*, 277-289.

Hayashida, H., Miyata, T., Yamawaki-Kataoka, Y., Honjo, T., Wels, J., and Blattner, F. (1984). Concerted evolution of the mouse immunoglobulin gamma chain genes. Embo J *3*, 2047-2053.

Hedges, S. B. (2002). The origin and evolution of model organisms. Nat Rev Genet *3*, 838-849.

Helm, B., Marsh, P., Vercelli, D., Padlan, E., Gould, H., and Geha, R. (1988). The mast cell binding site on human immunoglobulin E. Nature *331*, 180-183.

Helm, B. A., Ling, Y., Teale, C., Padlan, E. A., and Bruggemann, M. (1991). The nature and importance of the inter-epsilon chain disulfide bonds in human IgE. Eur J Immunol *21*, 1543-1548.

Henry, A. J. (1996). PhD thesis. University of London.

Henry, A. J., Cook, J. P., McDonnell, J. M., Mackay, G. A., Shi, J., Sutton, B. J., and Gould, H. J. (1997). Participation of the N-terminal region of Cepsilon3 in the binding of human IgE to its high-affinity receptor FcepsilonRI. Biochemistry *36*, 15568-15578.

Hillier, L. W., Miller, W., Birney, E., Warren, W., Hardison, R. C., Ponting, C. P., Bork, P., Burt, D. W., Groenen, M. A., Delany, M. E. *et al.* (2004). Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature *432*, 695-716.

Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998). SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics *14*, 378-379.

Hobbs, S. M., Jackson, L. E., and Hoadley, J. (1992). Interaction of aglycosyl immunoglobulins with the IgG Fc transport receptor from neonatal rat gut: comparison of deglycosylation by tunicamycin treatment and genetic engineering. Mol Immunol *29*, 949-956.

Hsu, E., Flajnik, M. F., and Du Pasquier, L. (1985). A third immunoglobulin class in amphibians. J Immunol *135*, 1998-2004.

Hubbard, S. C., and Ivatt, R. J. (1981). Synthesis and processing of asparagine-linked oligosaccharides. Annu Rev Biochem *50*, 555-583.

Huber, R., Deisenhofer, J., Colman, P. M., Matsushima, M., and Palm, W. (1976). Crystallographic structure studies of an IgG molecule and an Fc fragment. Nature *264*, 415-420.

Hughes-Jones, N. C., Feinstein, A., Richardson, N. E., Gorick, B. D., and Howard, J. C. (1984). C1 activation by immunoglobulin and immunoglobulin antibodies. Biochem Soc Trans *12*, 738-739.

Hughes, A. L. (1997). Rapid evolution of immunoglobulin superfamily C2 domains expressed in immune system cells. Mol Biol Evol *14*, 1-5.

Hulett, M. D., and Hogarth, P. M. (1994). Molecular basis of Fc receptor function. Adv Immunol *57*, 1-127.

Humphrey, B. D., Calvert, C. C., and Klasing, K. C. (2004). The ratio of full length IgY to truncated IgY in immune complexes affects macrophage phagocytosis and the acute phase response of mallard ducks (Anas platyrhynchos). Dev Comp Immunol *28*, 665-672.

Hunt, J. (2004). PhD Thesis. University of London.

Hunt, J., Beavil, R. L., Calvert, R. A., Gould, H. J., Sutton, B. J., and Beavil, A. J. (2005). Disulfide linkage controls the affinity and stoichiometry of IgE Fcepsilon3-4 binding to FcepsilonRI. J Biol Chem *280*, 16808-16814.

Ikari, K., Momohara, S., Nakamura, T., Hara, M., Yamanaka, H., Tomatsu, T., and Kamatani, N. (2006). Supportive evidence for a genetic association of the FCRL3 promoter polymorphism with rheumatoid arthritis. Ann Rheum Dis *65*, 671-673.

Ikeyama, S. (1987). Thermally induced disulfide bond exchanges in human IgE. Mol Immunol *24*, 231-237.

Ishizaka, T., Helm, B., Hakimi, J., Niebyl, J., Ishizaka, K., and Gould, H. (1986). Biological properties of a recombinant human immunoglobulin epsilon-chain fragment. Proc Natl Acad Sci U S A *83*, 8323-8327.

Ishizaka, T., Soto, C. S., and Ishizaka, K. (1973). Mechanisms of passive sensitization. 3. Number of IgE molecules and their receptor sites on human basophil granulocytes. J Immunol *111*, 500-511.

Janeway, C. (2001). Immunobiology : the immune system in health and disease, 5th edn (New York: Garland Pub.).

Jefferis, R., and Lund, J. (2002). Interaction sites on human IgG-Fc for FcgammaR: current models. Immunol Lett *82*, 57-65.

Johanson, Z., Long, J. A., Talent, J. A., Janvier, P., and Warren, J. W. (2006). Oldest coelacanth, from the Early Devonian of Australia. Biol Lett *2*, 443-446.

Jungi, T. W., and Hafner, S. (1986). Quantitative assessment of Fc receptor expression and function during in vitro differentiation of human monocytes to macrophages. Immunology *58*, 131-137.

Kaiser, P., Poh, T. Y., Rothwell, L., Avery, S., Balu, S., Pathania, U. S., Hughes, S., Goodchild, M., Morrell, S., Watson, M. *et al.* (2005). A genomic analysis of chicken cytokines and chemokines. J Interferon Cytokine Res *25*, 467-484.

Kao, F. T., and Puck, T. T. (1968). Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proc Natl Acad Sci U S A *60*, 1275-1281.

Karagiannis, S. N., Wang, Q., East, N., Burke, F., Riffard, S., Bracher, M. G., Thompson, R. G., Durham, S. R., Schwartz, L. B., Balkwill, F. R., and Gould, H. J. (2003). Activity of human monocytes in IgE antibody-dependent surveillance and killing of ovarian tumor cells. Eur J Immunol *33*, 1030-1040.

Kenten, J., Helm, B., Ishizaka, T., Cattini, P., and Gould, H. (1984). Properties of a human immunoglobulin epsilon-chain fragment synthesized in Escherichia coli. Proc Natl Acad Sci U S A *81*, 2955-2959.

Keown, M. B., Ghirlando, R., Mackay, G. A., Sutton, B. J., and Gould, H. J. (1997). Basis of the 1:1 stoichiometry of the high affinity receptor Fc epsilon RI-IgE complex. Eur Biophys J *25*, 471-476.

Keown, M. B., Ghirlando, R., Young, R. J., Beavil, A. J., Owens, R. J., Perkins, S. J., Sutton, B. J., and Gould, H. J. (1995). Hydrodynamic studies of a complex between the Fc fragment of human IgE and a soluble fragment of the Fc epsilon RI alpha chain. Proc Natl Acad Sci U S A *92*, 1841-1845.

Kepley, C., Craig, S., and Schwartz, L. (1994). Purification of human basophils by density and size alone. J Immunol Methods *175*, 1-9.

Kermani-Arab, V., Moll, T., Cho, B. R., Davis, W. C., and Lu, Y. S. (1976). Effects of IgY antibody on the development of Marek's disease. Avian Dis *20*, 32-41.

Kermode, J. C. (1988). Means for the assessment of radioligand quality and its importance in receptor-binding studies. Observations with radiolabelled formylmethionyl-leucyl-phenylalanine. Biochem J *252*, 521-528.

Kinet, J. P., Alcaraz, G., Leonard, A., Wank, S., and Metzger, H. (1985). Dissociation of the receptor for immunoglobulin E in mild detergents. Biochemistry *24*, 4117-4124.

Kobata, A. (1990). Function and pathology of the sugar chains of human immunoglobulin G. Glycobiology *1*, 5-8.

Kochi, Y., Yamada, R., Suzuki, A., Harley, J. B., Shirasawa, S., Sawada, T., Bae, S. C., Tokuhiro, S., Chang, X., Sekine, A. *et al.* (2005). A functional variant in FCRL3, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities. Nat Genet *37*, 478-485.

Kogut, M. H., Genovese, K. J., and Nisbet, D. J. (2001). Signal transduction pathways activated by engaging immunoglobulin Fc receptors on chicken heterophils. Dev Comp Immunol *25*, 639-646.

Koppenheffer, T. L., Chan, S. W., and Higgins, D. A. (1999). The complement system of the duck. Avian Pathol *28*, 17-25.

Koskela, K., Kohonen, P., Salminen, H., Uchida, T., Buerstedde, J. M., and Lassila, O. (2004). Identification of a novel cytokine-like transcript differentially expressed in avian gammadelta T cells. Immunogenetics *55*, 845-854.

Krapp, S., Mimura, Y., Jefferis, R., Huber, R., and Sondermann, P. (2003). Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. J Mol Biol *325*, 979-989.

Krovetz, H. S., VanDongen, H. M., and VanDongen, A. M. (1997). Atomic distance estimates from disulfides and high-affinity metal-binding sites in a K+ channel pore. Biophys J *72*, 117-126.

Kubo, R. T., and Benedict, A. A. (1969). Unusual conditions for crystallization of the Fc fragment of chicken IgG. J Immunol *102*, 1523-1525.

Kulczycki, A., Jr., and Metzger, H. (1974). The interaction of IgE with rat basophilic leukemia cells. II. Quantitative aspects of the binding reaction. J Exp Med *140*, 1676-1695.

Kumar, S., Tamura, K., and Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform *5*, 150-163.

Kumar, T. K., Gopalakrishna, K., Prasad, V. V., and Pandit, M. W. (1993). Multiple bands on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of proteins due to intermolecular disulfide cross-linking. Anal Biochem *213*, 226-228.

Kurosaki, T., and Ravetch, J. V. (1989). A single amino acid in the glycosyl phosphatidylinositol attachment domain determines the membrane topology of Fc gamma RIII. Nature *342*, 805-807.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Leslie, G. A., and Clem, L. W. (1969). Phylogen of immunoglobulin structure and function. 3. Immunoglobulins of the chicken. J Exp Med *130*, 1337-1352.

Leslie, G. A., and Clem, L. W. (1972). Phylogeny of immunoglobulin structure and function. VI. 17S, 7.5S and 5.7S anti-DNP of the turtle, Pseudamys scripta. J Immunol *108*, 1656-1664.

Leu, C. M., Davis, R. S., Gartland, L. A., Fine, W. D., and Cooper, M. D. (2005). FcRH1: an activation coreceptor on human B cells. Blood *105*, 1121-1126.

Linden, C. D., and Roth, T. F. (1978). IgG receptors on foetal chick yolk sac. J Cell Sci *33*, 317-328.

Lund, J., Takahashi, N., Hindley, S., Tyler, R., Goodall, M., and Jefferis, R. (1993). Glycosylation of human IgG subclass and mouse IgG2b heavy chains secreted by mouse J558L transfectoma cell lines as chimeric antibodies. Hum Antibodies Hybridomas *4*, 20-25.

Lund, J., Takahashi, N., Popplewell, A., Goodall, M., Pound, J. D., Tyler, R., King, D. J., and Jefferis, R. (2000). Expression and characterization of truncated forms of humanized L243 IgG1. Architectural features can influence synthesis of its oligosaccharide chains and affect superoxide production triggered through human Fcgamma receptor I. Eur J Biochem *267*, 7246-7257.

Lund, J., Tanaka, T., Takahashi, N., Sarmay, G., Arata, Y., and Jefferis, R. (1990). A protein structural change in aglycosylated IgG3 correlates with loss of huFc gamma R1 and huFc gamma R111 binding and/or activation. Mol Immunol *27*, 1145-1153.

Lundqvist, M. L., Middleton, D. L., Radford, C., Warr, G. W., and Magor, K. E. (2006). Immunoglobulins of the non-galliform birds: Antibody expression and repertoire in the duck. Dev Comp Immunol *30*, 93-100.

Maenaka, K., van der Merwe, P. A., Stuart, D. I., Jones, E. Y., and Sondermann, P. (2001). The human low affinity Fcgamma receptors IIa, IIb, and III bind IgG with fast kinetics and distinct thermodynamic properties. J Biol Chem *276*, 44898-44904.

Magor, K. E., Higgins, D. A., Middleton, D. L., and Warr, G. W. (1994). One gene encodes the heavy chains for three different forms of IgY in the duck. J Immunol *153*, 5549-5555.

Magor, K. E., Warr, G. W., Middleton, D., Wilson, M. R., and Higgins, D. A. (1992). Structural relationship between the two IgY of the duck, Anas platyrhynchos: molecular genetic evidence. J Immunol *149*, 2627-2633.

Malkinson, M. (1965). The transmission of passive immunity to Escherichia coli from mother to young in the domestic fowl (Gallus domesticus). Immunology *9*, 311-317.

Maltais, L. J., Lovering, R. C., Taranin, A. V., Colonna, M., Ravetch, J. V., Dalla-Favera, R., Burrows, P. D., Cooper, M. D., and Davis, R. S. (2006). New nomenclature for Fc receptor-like molecules. Nat Immunol *7*, 431-432.

Marchalonis, J., and Edelman, G. M. (1966). Phylogenetic origins of antibody structure. II. Immunoglobulins in the primary immune response of the bullfrog, Rana catesbiana. J Exp Med *124*, 901-913.

Masuda, M., and Roos, D. (1993). Association of all three types of Fc gamma R (CD64, CD32, and CD16) with a gamma-chain homodimer in cultured human monocytes. J Immunol *151*, 7188-7195.

Matre, R., Kleppe, G., and Tonder, O. (1981). Isolation and characterization of Fc gamma receptors from human placenta. Acta Pathol Microbiol Scand [C] *89*, 209-213.

Matsumiya, S., Yamaguchi, Y., Saito, J., Nagano, M., Sasakawa, H., Otaki, S., Satoh, M., Shitara, K., and Kato, K. (2007). Structural comparison of fucosylated and nonfucosylated fc fragments of human immunoglobulin g1. J Mol Biol *368*, 767-779.

Maurer, D., Fiebiger, S., Ebner, C., Reininger, B., Fischer, G. F., Wichlas, S., Jouvin, M. H., Schmitt-Egenolf, M., Kraft, D., Kinet, J. P., and Stingl, G. (1996). Peripheral blood dendritic cells express Fc epsilon RI as a complex composed of Fc epsilon RI alpha- and Fc epsilon RI gamma-chains and can use this receptor for IgE-mediated allergen presentation. J Immunol *157*, 607-616.

Maynard Smith, J. (1998). Evolutionary genetics, 2nd edn (Oxford: Oxford University Press,).

Mayr, G., Pohl, B., and Peters, D. S. (2005). A well-preserved Archaeopteryx specimen with theropod features. Science *310*, 1483-1486.

McCloskey, N., Hunt, J., Beavil, R. L., Jutton, M. R., Grundy, G. J., Girardi, E., Fabiane, S. M., Fear, D. J., Conrad, D. H., Sutton, B. J., and Gould, H. J. (2007). Soluble CD23 monomers inhibit and oligomers stimulate IGE synthesis in human B cells. J Biol Chem.

McConahey, P. J., and Dixon, F. J. (1980). Radioiodination of proteins by the use of the chloramine-T method. Methods Enzymol *70*, 210-213.

McCool, D., Birshtein, B. K., and Painter, R. H. (1985). Structural requirements of immunoglobulin G for binding to the Fc gamma receptors of the human tumor cell lines U937, HL-60, ML-1, and K562. J Immunol *135*, 1975-1980.

McDonnell, J. M., Calvert, R., Beavil, R. L., Beavil, A. J., Henry, A. J., Sutton, B. J., Gould, H. J., and Cowburn, D. (2001). The structure of the IgE Cepsilon2 domain and its role in stabilizing the complex with its high-affinity receptor FcepsilonRIalpha. Nat Struct Biol *8*, 437-441.

Mechetina, L. V., Najakshin, A. M., Volkova, O. Y., Guselnikov, S. V., Faizulin, R. Z., Alabyev, B. Y., Chikaev, N. A., Vinogradova, M. S., and Taranin, A. V. (2002). FCRL, a novel member of the leukocyte Fc receptor family possesses unique structural features. Eur J Immunol *32*, 87-96.

Melson, B. E., Sluss, P. M., and Reichert, L. E., Jr. (1987). Effects of different batches of 125iodine on properties of 125I-hFSH and characteristics of radioligand-receptor assays. Anal Biochem *160*, 434-439.

Miller, I., Hatzivassiliou, G., Cattoretti, G., Mendelsohn, C., and Dalla-Favera, R. (2002). IRTAs: a new family of immunoglobulinlike receptors differentially expressed in B cells. Blood *99*, 2662-2669.

Miller, K. L., Duchemin, A. M., and Anderson, C. L. (1996). A novel role for the Fc receptor gamma subunit: enhancement of Fc gamma R ligand affinity. J Exp Med *183*, 2227-2233.

Miller, L., Blank, U., Metzger, H., and Kinet, J. P. (1989). Expression of high-affinity binding of human immunoglobulin E by transfected cells. Science *244*, 334-337.

Milstein, C., and Pink, J. R. (1970). Structure and evolution of immunoglobulins. Prog Biophys Mol Biol *21*, 209-263.

Mimura, Y., Church, S., Ghirlando, R., Ashton, P. R., Dong, S., Goodall, M., Lund, J., and Jefferis, R. (2000). The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms. Mol Immunol *37*, 697-706.

Mimura, Y., Sondermann, P., Ghirlando, R., Lund, J., Young, S. P., Goodall, M., and Jefferis, R. (2001). Role of oligosaccharide residues of IgG1-Fc in Fc gamma RIIb binding. J Biol Chem *276*, 45539-45547.

Morkowski, J., and Nowak, J. (1981). Preliminary results of the isolation of chicken FcIgG receptor. Arch Immunol Ther Exp (Warsz) *29*, 611-614.

Morrison, S. L., Mohammed, M. S., Wims, L. A., Trinh, R., and Etches, R. (2002). Sequences in antibody molecules important for receptor-mediated transport into the chicken egg yolk. Mol Immunol *38*, 619-625.

Moscovici, M. G., Siegel, M. L., and Moscovici, C. (1989). Avian erythroblastosis virus transforms a novel mast cell-basophil precursor target in the Japanese quail. J Virol *63*, 2335-2339.

Murphy, P. M. (1993). Molecular mimicry and the generation of host defense protein diversity. Cell *72*, 823-826.

Mussmann, R., Wilson, M., Marcuz, A., Courtet, M., and Du Pasquier, L. (1996). Membrane exon sequences of the three Xenopus Ig classes explain the evolutionary origin of mammalian isotypes. Eur J Immunol *26*, 409-414.

Nettleton, M. Y., and Kochan, J. P. (1995). Role of glycosylation sites in the IgE Fc molecule. Int Arch Allergy Immunol *107*, 328-329.

Nezlin, R. S., Zagyansky, Y. A., Kaivarainen, A. I., and Stefani, D. V. (1973). Properties of myeloma immunoglobulin E(Yu). Chemical, fluorescence polarisation and spin-labeled studies. Immunochemistry *10*, 681-688.

Nimmerjahn, F., Bruhns, P., Horiuchi, K., and Ravetch, J. V. (2005). FcgammaRIV: a novel FcR with distinct IgG subclass specificity. Immunity *23*, 41-51.

Nimmerjahn, F., and Ravetch, J. V. (2006). Fcgamma receptors: old friends and new family members. Immunity *24*, 19-28.

Noll, F., Lutsch, G., and Bielka, H. (1982). Structure of IgG and IgY molecules in ribosome-antibody complexes as studied by electron microscopy. Immunol Lett *4*, 117-123.

Nose, M., Takano, R., Nakamura, S., Arata, Y., and Kyogoku, M. (1990). Recombinant Fc of human IgG1 prepared in an Escherichia coli system escapes recognition by macrophages. Int Immunol 2, 1109-1112.

O'Donnell, I. J., Frangione, B., and Porter, R. R. (1970). The disulphide bonds of the heavy chain of rabbit immunoglobulin G. Biochem J *116*, 261-268.

Oi, V. T., Vuong, T. M., Hardy, R., Reidler, J., Dangle, J., Herzenberg, L. A., and Stryer, L. (1984). Correlation between segmental flexibility and effector function of antibodies. Nature *307*, 136-140.

Otten, M. A., and van Egmond, M. (2004). The Fc receptor for IgA (FcalphaRI, CD89). Immunol Lett *92*, 23-31.

Padlan, E. A., and Davies, D. R. (1986). A model of the Fc of immunoglobulin E. Mol Immunol *23*, 1063-1075.

Paetz, A., Sack, M., Thepen, T., Tur, M. K., Bruell, D., Finnern, R., Fischer, R., and Barth, S. (2005). Recombinant soluble human Fcgamma receptor I with picomolar affinity for immunoglobulin G. Biochem Biophys Res Commun *338*, 1811-1817.

Parham, P. (1995). Antibody structure. The duck's dilemma. Nature *374*, 16-17.

Parvari, R., Avivi, A., Lentner, F., Ziv, E., Tel-Or, S., Burstein, Y., and Schechter, I. (1988). Chicken immunoglobulin gamma-heavy chains: limited VH gene repertoire, combinatorial diversification by D gene segments and evolution of the heavy chain locus. Embo J *7*, 739-744.

Perez-Montfort, R., Kinet, J. P., and Metzger, H. (1983). A previously unrecognized subunit of the receptor for immunoglobulin E. Biochemistry *22*, 5722-5728.

Perkins, S. J., Nealis, A. S., Sutton, B. J., and Feinstein, A. (1991). Solution structure of human and mouse immunoglobulin M by synchrotron X-ray scattering and molecular graphics modelling. A possible mechanism for complement activation. J Mol Biol *221*, 1345-1366.

Porter, R. R. (1959). The hydrolysis of rabbit y-globulin and antibodies with crystalline papain. Biochem J *73*, 119-126.

Pouyaud, L., Wirjoatmodjo, S., Rachmatika, I., Tjakrawidjaja, A., Hadiaty, R., and Hadie, W. (1999). [A new species of coelacanth. Genetic and morphologic proof]. C R Acad Sci III *322*, 261-267.

Price, N. E., Price, N. C., Kelly, S. M., and McDonnell, J. M. (2005). The key role of protein flexibility in modulating IgE interactions. J Biol Chem *280*, 2324-2330.

Pruzansky, J. J., Grammer, L. C., Patterson, R., and Roberts, M. (1983). Dissociation of IgE from receptors on human basophils. I. Enhanced passive sensitization for histamine release. J Immunol *131*, 1949-1953.

Ptitsyn, O. B. (1995). How the molten globule became. Trends Biochem Sci *20*, 376-379.

Puck, T. T., Cieciura, S. J., and Robinson, A. (1958). Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. J Exp Med *108*, 945-956.

Qureshi, M. A., Heggen, C. L., and Hussain, I. (2000). Avian macrophage: effector functions in health and disease. Dev Comp Immunol *24*, 103-119.

Qureshi, M. A., Miller, L., Lillehoj, H. S., and Ficken, M. D. (1990). Establishment and characterization of a chicken mononuclear cell line. Vet Immunol Immunopathol *26*, 237-250.

Ra, C., Jouvin, M. H., Blank, U., and Kinet, J. P. (1989). A macrophage Fc gamma receptor and the mast cell receptor for IgE share an identical subunit. Nature *341*, 752-754.

Radaev, S., and Sun, P. D. (2001). Recognition of IgG by Fcgamma receptor. The role of Fc glycosylation and the binding of peptide inhibitors. J Biol Chem *276*, 16478-16483.

Ravetch, J. V., and Kinet, J. P. (1991). Fc receptors. Annu Rev Immunol *9*, 457-492.

Raychaudhuri, G., McCool, D., and Painter, R. H. (1985). Human IgG1 and its Fc fragment bind with different affinities to the Fc receptors on the human U937, HL-60 and ML-1 cell lines. Mol Immunol *22*, 1009-1019.

Reisz, R. R., and Muller, J. (2004). Molecular timescales and the fossil record: a paleontological perspective. Trends Genet *20*, 237-241.

Reynaud, C. A., Dahan, A., Anquez, V., and Weill, J. C. (1989). Somatic hyperconversion diversifies the single Vh gene of the chicken with a high incidence in the D region. Cell *59*, 171-183.

Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979). Ellman's reagent: 5,5'-dithiobis(2-nitrobenzoic acid)--a reexamination. Anal Biochem *94*, 75-81.

Riener, C. K., Kada, G., and Gruber, H. J. (2002). Quick measurement of protein sulfhydryls with Ellman's reagent and with 4,4'-dithiodipyridine. Anal Bioanal Chem *373*, 266-276.

Rose, M. E., Ogilvie, B. M., and Bradley, J. W. (1980). Intestinal mast cell response in rats and chickens to coccidiosis, with some properties of chicken mast cells. Int Arch Allergy Appl Immunol *63*, 21-29.

Rosenthal, H. E. (1967). A graphic method for the determination and presentation of binding parameters in a complex system. Anal Biochem *20*, 525-532.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A *74*, 5463-5467.

Savan, R., Aman, A., Sato, K., Yamaguchi, R., and Sakai, M. (2005). Discovery of a new class of immunoglobulin heavy chain from fugu. Eur J Immunol.

Schweitzer, M. H., Wittmeyer, J. L., and Horner, J. R. (2007). Soft tissue and cellular preservation in vertebrate skeletal elements from the Cretaceous to the present. Proc Biol Sci *274*, 183-197.

Schweitzer, M. H., Wittmeyer, J. L., Horner, J. R., and Toporski, J. K. (2005). Soft-tissue vessels and cellular preservation in Tyrannosaurus rex. Science *307*, 1952-1955.

Shi, J., Ghirlando, R., Beavil, R. L., Beavil, A. J., Keown, M. B., Young, R. J., Owens, R. J., Sutton, B. J., and Gould, H. J. (1997). Interaction of the low-affinity receptor CD23/Fc epsilonRII lectin domain with the Fc epsilon3-4 fragment of human immunoglobulin E. Biochemistry *36*, 2112-2122.

Shields, R. L., Lai, J., Keck, R., O'Connell, L. Y., Hong, K., Meng, Y. G., Weikert, S. H., and Presta, L. G. (2002). Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J Biol Chem *277*, 26733-26740.

Shimizu, M., Nagashima, H., and Hashimoto, K. (1993). Comparative studies in molecular stability of immunoglobulin G from different species. Comp Biochem Physiol B *106*, 255-261.

Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M. *et al.* (2003). The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem *278*, 3466-3473.

Shopes, B., Weetall, M., Holowka, D., and Baird, B. (1990). Recombinant human IgG1-murine IgE chimeric Ig. Construction, expression, and binding to human Fc gamma receptors. J Immunol *145*, 3842-3848.

Smith, J. L. B. (1939). Nature *143*, 455-456.

Sondermann, P., Huber, R., Oosthuizen, V., and Jacob, U. (2000). The 3.2-A crystal structure of the human IgG1 Fc fragment-Fc gammaRIII complex. Nature *406*, 267-273.

Sondermann, P., and Oosthuizen, V. (2002). X-ray crystallographic studies of IgG-Fc gamma receptor interactions. Biochem Soc Trans *30*, 481-486.

Stafford, J. L., Wilson, M., Nayak, D., Quiniou, S. M., Clem, L. W., Miller, N. W., and Bengten, E. (2006). Identification and characterization of a FcR homolog in an ectothermic vertebrate, the channel catfish (Ictalurus punctatus). J Immunol *177*, 2505-2517.

Stephens, P. E., and Cockett, M. I. (1989). The construction of a highly efficient and versatile set of mammalian expression vectors. Nucleic Acids Res *17*, 7110.

Sundstrom, C., and Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer *17*, 565-577.

Suzuki, N., and Lee, Y. C. (2004). Site-specific N-glycosylation of chicken serum IgG. Glycobiology *14*, 275-292.

Suzuki, Y., Ra, C., Saito, K., Horikoshi, S., Hasegawa, S., Tsuge, T., Okumura, K., and Tomino, Y. (1999). Expression and physical association of Fc alpha receptor and Fc receptor gamma chain in human mesangial cells. Nephrol Dial Transplant *14*, 1117-1123.

Takai, T., Li, M., Sylvestre, D., Clynes, R., and Ravetch, J. V. (1994). FcR gamma chain deletion results in pleiotrophic effector cell defects. Cell *76*, 519-529.

Takatsu, K., Ishizaka, T., and Ishizaka, K. (1975). Biologic significance of disulfide bonds in human IgE molecules. J Immunol *114*, 1838-1845.

Tao, M. H., and Morrison, S. L. (1989). Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. J Immunol *143*, 2595-2601.

Taylor, A. I., Gould, H. J., Sutton, B. J., and Calvert, R. A. (2007). The first avian Ig-like Fc receptor family member combines features of mammalian FcR and FCRL. Immunogenetics *59*, 323-328.

Teillaud, J. L., Bouchard, C., Astier, A., Teillaud, C., Tartour, E., Michon, J., Galinha, A., Moncuit, J., Mazieres, N., Spagnoli, R., and *et al.* (1994). Natural and recombinant soluble low-affinity Fc gamma R: detection, purification, and functional activities. Immunomethods *4*, 48-64.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22, 4673-4680.

Tischenko, V. M., Abramov, V. M., and Zav'yalov, V. P. (1998). Investigation of the cooperative structure of Fc fragments from myeloma immunoglobulin G. Biochemistry 37, 5576-5581.

Tressler, R. L., and Roth, T. F. (1987). IgG receptors on the embryonic chick yolk sac. J Biol Chem *262*, 15406-15412.

Umana, P., Jean-Mairet, J., Moudry, R., Amstutz, H., and Bailey, J. E. (1999). Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. Nat Biotechnol *17*, 176-180.

Umemura, T., Ota, M., Hamano, H., Katsuyama, Y., Kiyosawa, K., and Kawa, S. (2006). Genetic association of Fc receptor-like 3 polymorphisms with autoimmune pancreatitis in Japanese patients. Gut *55*, 1367-1368.

Unkeless, J. C., and Eisen, H. N. (1975). Binding of monomeric immunoglobulins to Fc receptors of mouse macrophages. J Exp Med *142*, 1520-1533.

van Vugt, M. J., Heijnen, A. F., Capel, P. J., Park, S. Y., Ra, C., Saito, T., Verbeek, J. S., and van de Winkel, J. G. (1996). FcR gamma-chain is essential for both surface expression and function of human Fc gamma RI (CD64) in vivo. Blood *87*, 3593-3599.

van Vugt, M. J., Reefman, E., Zeelenberg, I., Boonen, G., Leusen, J. H., and van de Winkel, J. G. (1999). The alternatively spliced CD64 transcript FcgammaRIb2 does not specify a surface-expressed isoform. Eur J Immunol *29*, 143-149.

Vargas, A. O., and Fallon, J. F. (2005). Birds have dinosaur wings: The molecular evidence. J Exp Zoolog B Mol Dev Evol *304*, 86-90.

Vercelli, D., Helm, B., Marsh, P., Padlan, E., Geha, R. S., and Gould, H. (1989). The B-cell binding site on human immunoglobulin E. Nature *338*, 649-651.

Vermeer, A. W., and Norde, W. (2000). The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein. Biophys J *78*, 394-404.

Vernersson, M., Aveskogh, M., and Hellman, L. (2004). Cloning of IgE from the echidna (Tachyglossus aculeatus) and a comparative analysis of epsilon chains from all three extant mammalian lineages. Dev Comp Immunol *28*, 61-75.

Vernersson, M., Aveskogh, M., Munday, B., and Hellman, L. (2002). Evidence for an early appearance of modern post-switch immunoglobulin isotypes in mammalian evolution (II); cloning of IgE, IgG1 and IgG2 from a monotreme, the duck-billed platypus, Ornithorhynchus anatinus. Eur J Immunol *32*, 2145-2155.

Viertlboeck, B. C., Crooijmans, R. P., Groenen, M. A., and Gobel, T. W. (2004). Chicken Ig-like receptor B2, a member of a multigene family, is mainly expressed on B lymphocytes, recruits both Src homology 2 domain containing protein tyrosine phosphatase (SHP)-1 and SHP-2, and inhibits proliferation. J Immunol *173*, 7385-7393.

Viertlboeck, B. C., Habermann, F. A., Schmitt, R., Groenen, M. A., Du Pasquier, L., and Gobel, T. W. (2005). The chicken leukocyte receptor complex: a highly diverse multigene family encoding at least six structurally distinct receptor types. J Immunol *175*, 385-393.

Viertlboeck, B. C., Schweinsberg, S., Hanczaruk, M. A., Schmitt, R., Du Pasquier, L., Herberg, F. W., and Gobel, T. W. (2007). The chicken leukocyte receptor complex encodes a primordial, activating, high-affinity IgY Fc receptor. Proc Natl Acad Sci U S A *104*, 11718-11723.

Walker, J. M. (2005). The proteomics protocols handbook (Totowa, N.J.: Humana Press).

Walker, M. R., Lund, J., Thompson, K. M., and Jefferis, R. (1989). Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing Fc gamma RI and/or Fc gamma RII receptors. Biochem J *259*, 347-353.

Wan, T., Beavil, R. L., Fabiane, S. M., Beavil, A. J., Sohi, M. K., Keown, M., Young, R. J., Henry, A. J., Owens, R. J., Gould, H. J., and Sutton, B. J. (2002). The crystal structure of IgE Fc reveals an asymmetrically bent conformation. Nat Immunol *3*, 681-686.

Wang, B., Rieger, A., Kilgus, O., Ochiai, K., Maurer, D., Fodinger, D., Kinet, J. P., and Stingl, G. (1992). Epidermal Langerhans cells from normal human skin bind monomeric IgE via Fc epsilon RI. J Exp Med *175*, 1353-1365.

Warr, G. W., Magor, K. E., and Higgins, D. A. (1995). IgY: clues to the origins of modern antibodies. Immunol Today *16*, 392-398.

Weil, G. J., Leiserson, W. M., and Chused, T. M. (1983). Isolation of human basophils by flow microfluorometry. J Immunol Methods *58*, 359-363.

West, A. P., Jr., Herr, A. B., and Bjorkman, P. J. (2004). The chicken yolk sac IgY receptor, a functional equivalent of the mammalian MHC-related Fc receptor, is a phospholipase A2 receptor homolog. Immunity *20*, 601-610.

Wiegand, T. W., Williams, P. B., Dreskin, S. C., Jouvin, M. H., Kinet, J. P., and Tasset, D. (1996). High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor I. J Immunol *157*, 221-230.

Wieland, W. H., Orzaez, D., Lammers, A., Parmentier, H. K., Verstegen, M. W., and Schots, A. (2004). A functional polymeric immunoglobulin receptor in chicken (Gallus gallus) indicates ancient role of secretory IgA in mucosal immunity. Biochem J *380*, 669-676.

Wilson, A. B., and Heller, E. D. (1976). Passive sensitization of tissue cells. V. The detection of chicken antibodies cytophilic for basophils and eosinophils. Int Arch Allergy Appl Immunol *51*, 68-79.

Wines, B. D., Trist, H. M., Ramsland, P. A., and Hogarth, P. M. (2006). A common site of the Fc receptor gamma subunit interacts with the unrelated immunoreceptors FcalphaRI and FcepsilonRI. J Biol Chem *281*, 17108-17113.

Woof, J. M (1985). PhD Thesis, University of Sheffield.

Woof, J. M., and Burton, D. R. (2004). Human antibody-Fc receptor interactions illuminated by crystal structures. Nat Rev Immunol *4*, 89-99.

Woof, J. M., and Kerr, M. A. (2006). The function of immunoglobulin A in immunity. J Pathol *208*, 270-282.

Woolhiser, M. R., Okayama, Y., Gilfillan, A. M., and Metcalfe, D. D. (2001). IgG-dependent activation of human mast cells following up-regulation of FcgammaRI by IFN-gamma. Eur J Immunol *31*, 3298-3307.

Wright, A., and Morrison, S. L. (1997). Effect of glycosylation on antibody function: implications for genetic engineering. Trends Biotechnol *15*, 26-32.

Wu, J., Cherwinski, H., Spies, T., Phillips, J. H., and Lanier, L. L. (2000). DAP10 and DAP12 form distinct, but functionally cooperative, receptor complexes in natural killer cells. J Exp Med *192*, 1059-1068.

Wurzburg, B. A., Garman, S. C., and Jardetzky, T. S. (2000). Structure of the human IgE-Fc C epsilon 3-C epsilon 4 reveals conformational flexibility in the antibody effector domains. Immunity *13*, 375-385.

Wurzburg, B. A., and Jardetzky, T. S. (2002). Structural insights into the interactions between human IgE and its high affinity receptor FcepsilonRI. Mol Immunol *38*, 1063-1072.

Xia, J., Radford, C., Guo, X., and Magor, K. E. (2006). Immune gene discovery by expressed sequence tag analysis of spleen in the duck (Anas platyrhynchos). Dev Comp Immunol.

Young, R. J. (1994). PhD Thesis. University of London.

Young, R. J., Owens, R. J., Mackay, G. A., Chan, C. M., Shi, J., Hide, M., Francis, D. M., Henry, A. J., Sutton, B. J., and Gould, H. J. (1995). Secretion of recombinant human IgE-Fc by mammalian cells and biological activity of glycosylation site mutants. Protein Eng *8*, 193-199.

Zaccone, P., Fehervari, Z., Phillips, J. M., Dunne, D. W., and Cooke, A. (2006). Parasitic worms and inflammatory diseases. Parasite Immunol *28*, 515-523.

Zdobnov, E. M., and Apweiler, R. (2001). InterProScan--an integration platform for the signature-recognition methods in InterPro. Bioinformatics *17*, 847-848.

Zhao, Y., Pan-Hammarstrom, Q., Yu, S., Wertz, N., Zhang, X., Li, N., Butler, J. E., and Hammarstrom, L. (2006). Identification of IgF, a hinge-region-containing Ig class, and IgD in Xenopus tropicalis. Proc Natl Acad Sci U S A *103*, 12087-12092.

Zheng, Y., Shopes, B., Holowka, D., and Baird, B. (1992). Dynamic conformations compared for IgE and IgG1 in solution and bound to receptors. Biochemistry *31*, 7446-7456.

Appendices

Appendix 1: Supplementary figures

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1     MSPLVSSLLL LAALPGLMAA VTLDESGGGL QTPGGGLSLV CKASGFTFSS
51    YNMGWVRQAP GKGLEFVAAI SSTGSGTNYG SAVKGRATIS RDNGQSTLRL
101   QLNNLRAEDT GTYYCARDLG YGDLYAGQID AWGHGTEVIV SSASPTSPPR
151   LYPLSACCSD SAVPPAVGCL LSPSSAGGIS WEGSGGTAVA GRVSGTPVKL
201   SFVRLSPGEK RKSFVCSAAP GGALLKKEVQ VCRVDPVPPV APEVQVLHPS
251   SCTPSQSESV ELLCLVTGFS PASAEVEWLV DGVGGLLVAS QSPAVRSGST
301   YSLSSRVNVS GTDWREGKSY SCRVRHPATN TVVEDHVKGC PDGAQSCSPI
351   QLYAIPPSPG ELYISLDAKL RCLVVNLPSD SSLSVTWTRE KSGNLRPDPM
401   VLQEHFNGTY SASSAVPVST QDWLSGERFT CTVQHEELPL PLSKSVYRNT
451   GPTTPPLIYP FAPHPEELSL SRVTLSCLVR GFRPRDIEIR WLRDHRAVPA
501   TEFVTTAVLP EERTANGAGG DGDTFFVYSK MSVETAKWNG GTVFACMAVH
551   EALPMRFSQR TLQKQAGK
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Figure A1.1
Upsilon heavy chain amino acid sequence numbering system

A complete upsilon heavy chain sequence was assembled (including the signal peptide) using published cDNA sequences; accession numbers M30350 (Reynaud *et al.*, 1989) and X07174 (Parvari *et al.*, 1988). Amino acid numbering used in this thesis begins with the first methionine in the translated sequence.

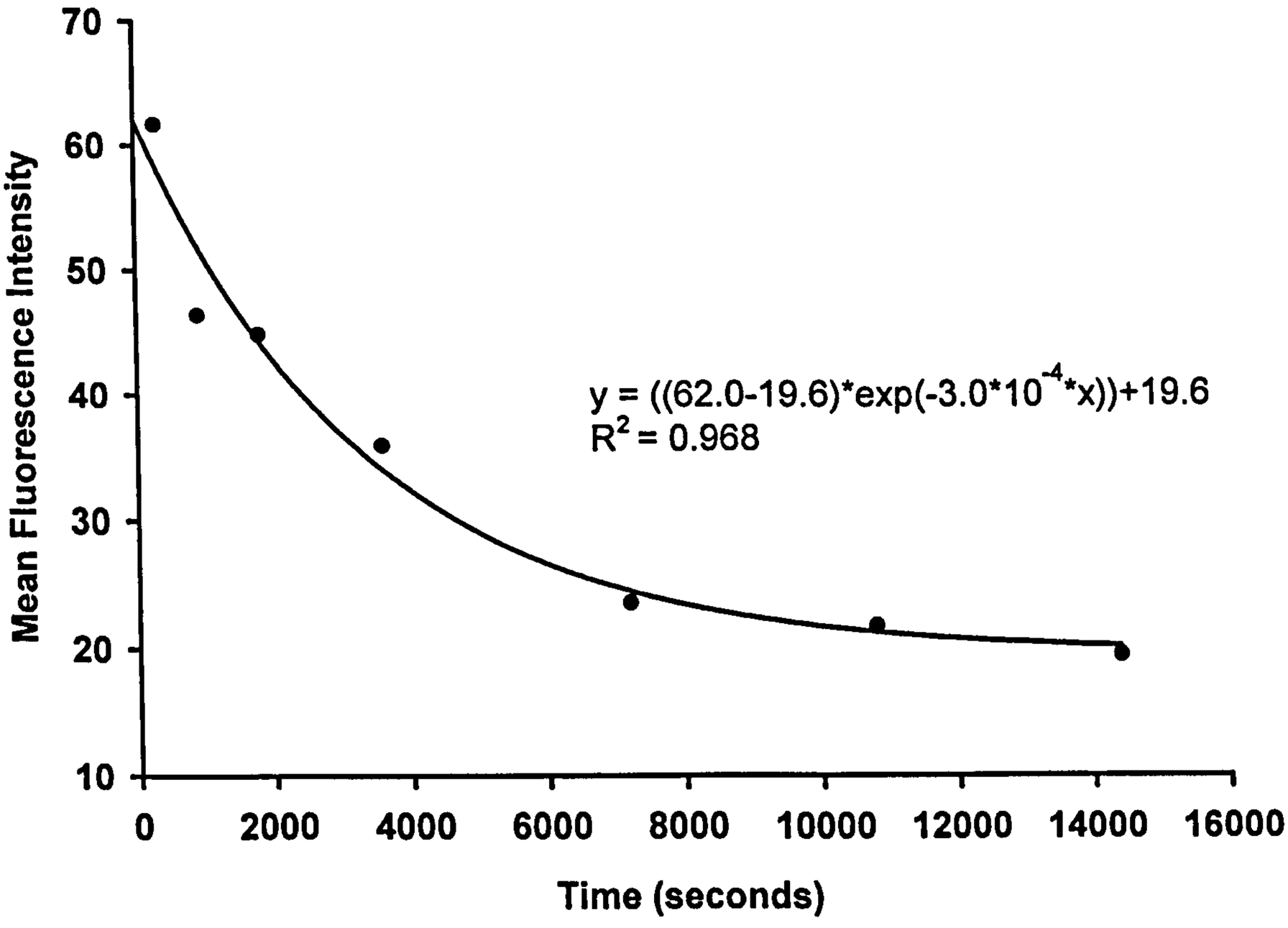

Figure A1.2
Dissociation of IgY from chicken monocytes monitored using flow cytometry

Graph showing mean fluorescence intensity of MQ-NCSU cells saturated with 50nM biotinylated chicken serum IgY then incubated with an excess of unlabelled IgY for the times shown before being stained with streptavidin-RPE for 20 minutes. All steps were carried out at 4°C. The data were fitted to a modified exponential decay model (see chapter 2) using SigmaPlot 6.0 (SPSS).

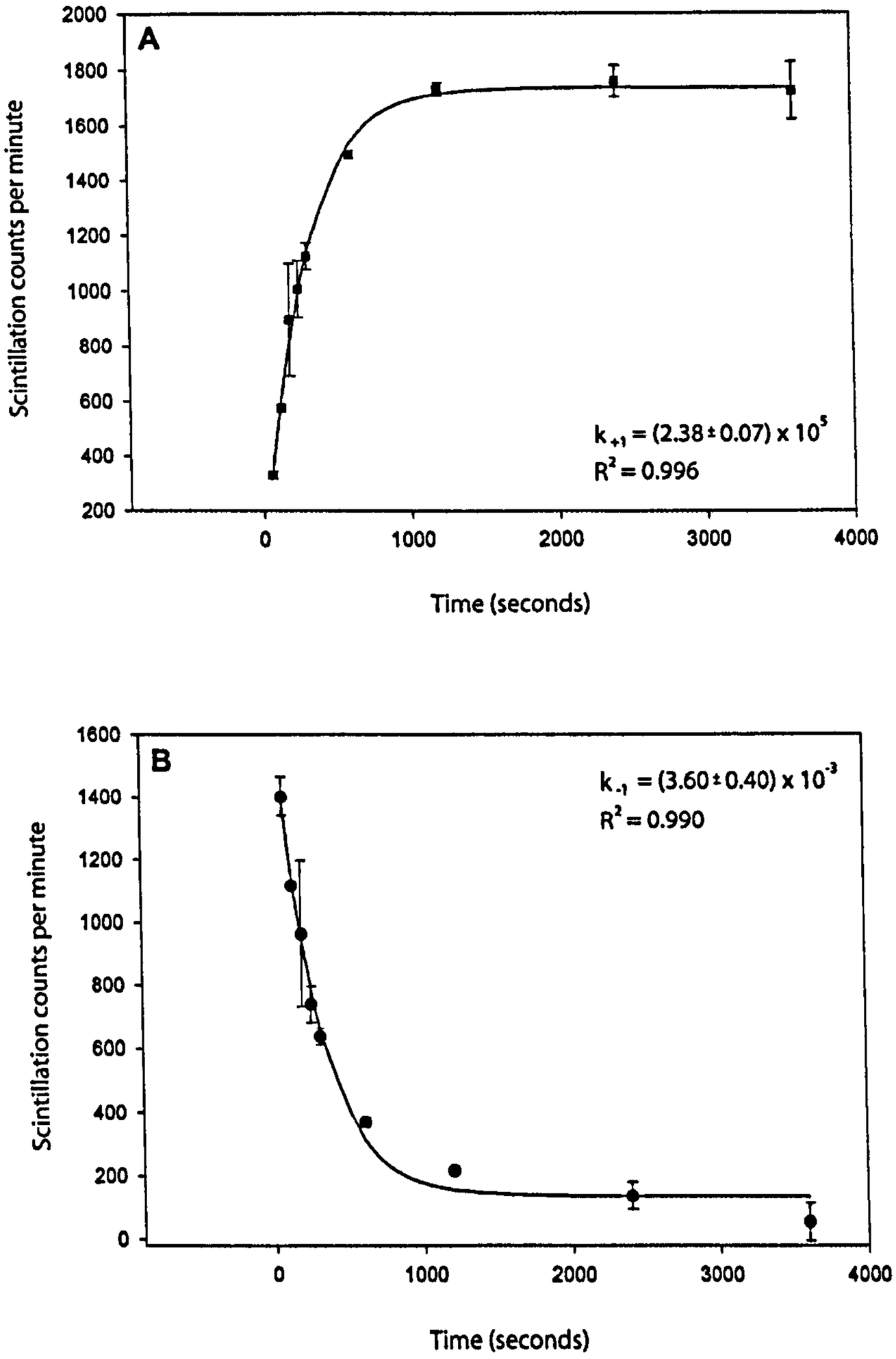

Figure A1.3
Kinetics of IgY-Fc Cυ2-4 [N308Q] binding to chicken monocytes

Cell binding assays showing experiments used to determine (A) association rate constant (k_{+1}) and (B) dissociation rate constant (k_{-1}) for recombinant IgY-Fc Cυ2-4 [N308Q] binding to MQ-NCSU cells. Assays were performed in duplicate and data is shown as mean ± s.e.m.
In (A) cells were incubated in 10nM ^{125}I-labelled ligand. In (B) cells were first saturated with ^{125}I-labelled ligand then washed and incubated with excess unlabelled ligand. In both experiments, 10^6 cells were removed at the times shown, spun through phthalate oil and their radioactivity measured using an automatic gamma counter. Regression analysis was used to fit the data to non-linear models (see chapter 2). The R^2 value provides a measure of how well the models fit the data, with 1 being a perfect fit.

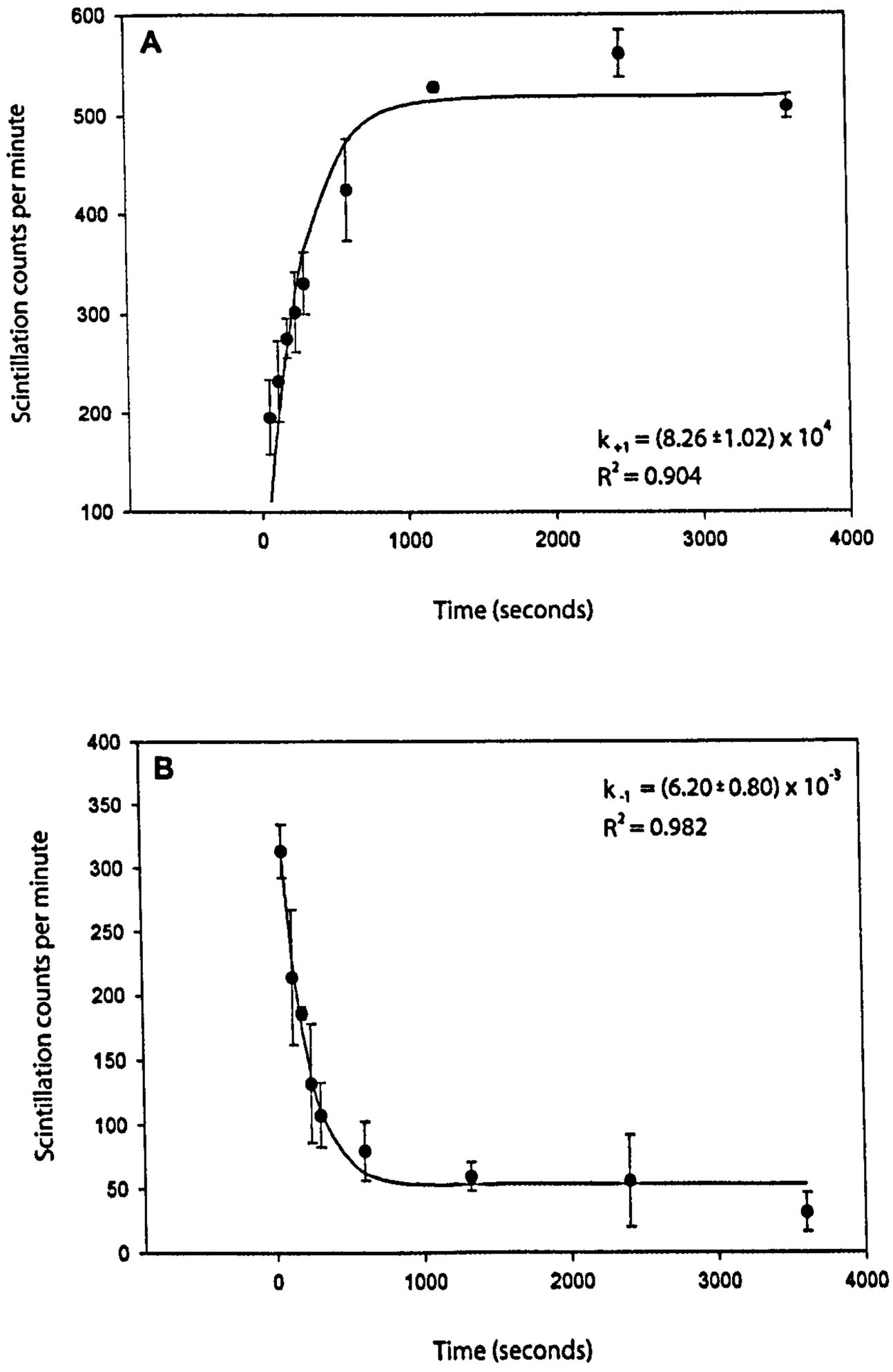

Figure A1.4
Kinetics of IgY-Fc Cυ2-4 [N308Q N407Q] binding to chicken monocytes

Cell binding assays showing experiments used to determine (A) association rate constant (k_{+1}) and (B) dissociation rate constant (k_{-1}) for recombinant IgY-Fc Cυ2-4 [N308Q N407Q] binding to MQ-NCSU cells. Assays were performed in duplicate and data is shown as mean ± s.e.m.
In (A) cells were incubated in 10nM ^{125}I-labelled ligand. In (B) cells were first saturated with ^{125}I-labelled ligand then washed and incubated with excess unlabelled ligand. In both experiments, 10^6 cells were removed at the times shown, spun through phthalate oil and their radioactivity measured using an automatic gamma counter. Regression analysis was used to fit the data to non-linear models (see chapter 2). The R^2 value provides a measure of how well the models fit the data, with 1 being a perfect fit.

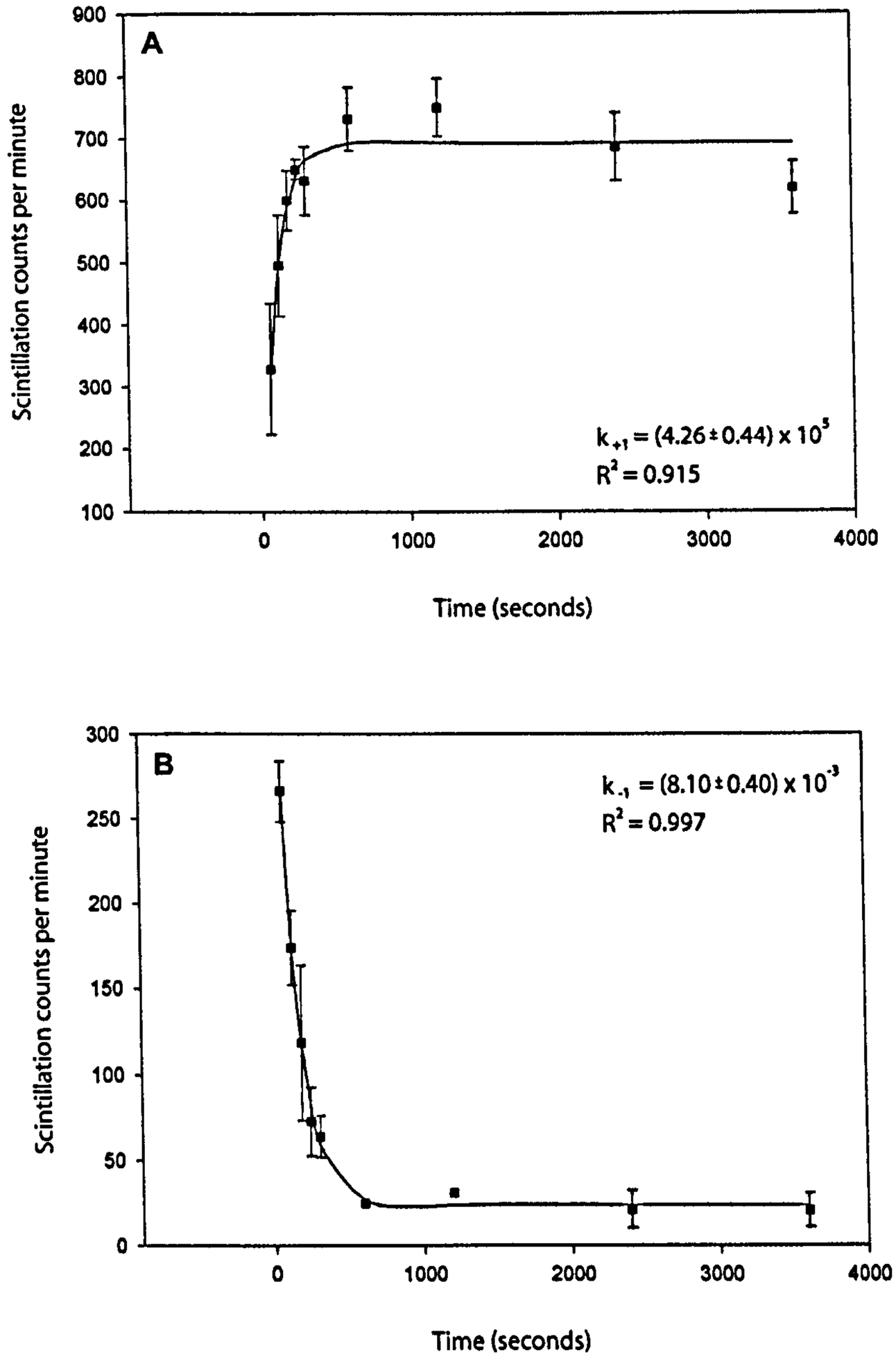

Figure A1.5
Kinetics of IgY-Fc Cυ3-4 [wt] binding to chicken monocytes

Cell binding assays showing experiments used to determine (A) association rate constant (k_{+1}) and (B) dissociation rate constant (k_{-1}) for recombinant IgY-Fc Cυ3-4 [wt] binding to MQ-NCSU cells. Assays were performed in duplicate and data is shown as mean ± s.e.m.
In (A) cells were incubated in 10nM ^{125}I-labelled ligand. In (B) cells were first saturated with ^{125}I-labelled ligand then washed and incubated with excess unlabelled ligand. In both experiments, 10^6 cells were removed at the times shown, spun through phthalate oil and their radioactivity measured using an automatic gamma counter. Regression analysis was used to fit the data to non-linear models (see chapter 2). The R^2 value provides a measure of how well the models fit the data, with 1 being a perfect fit.

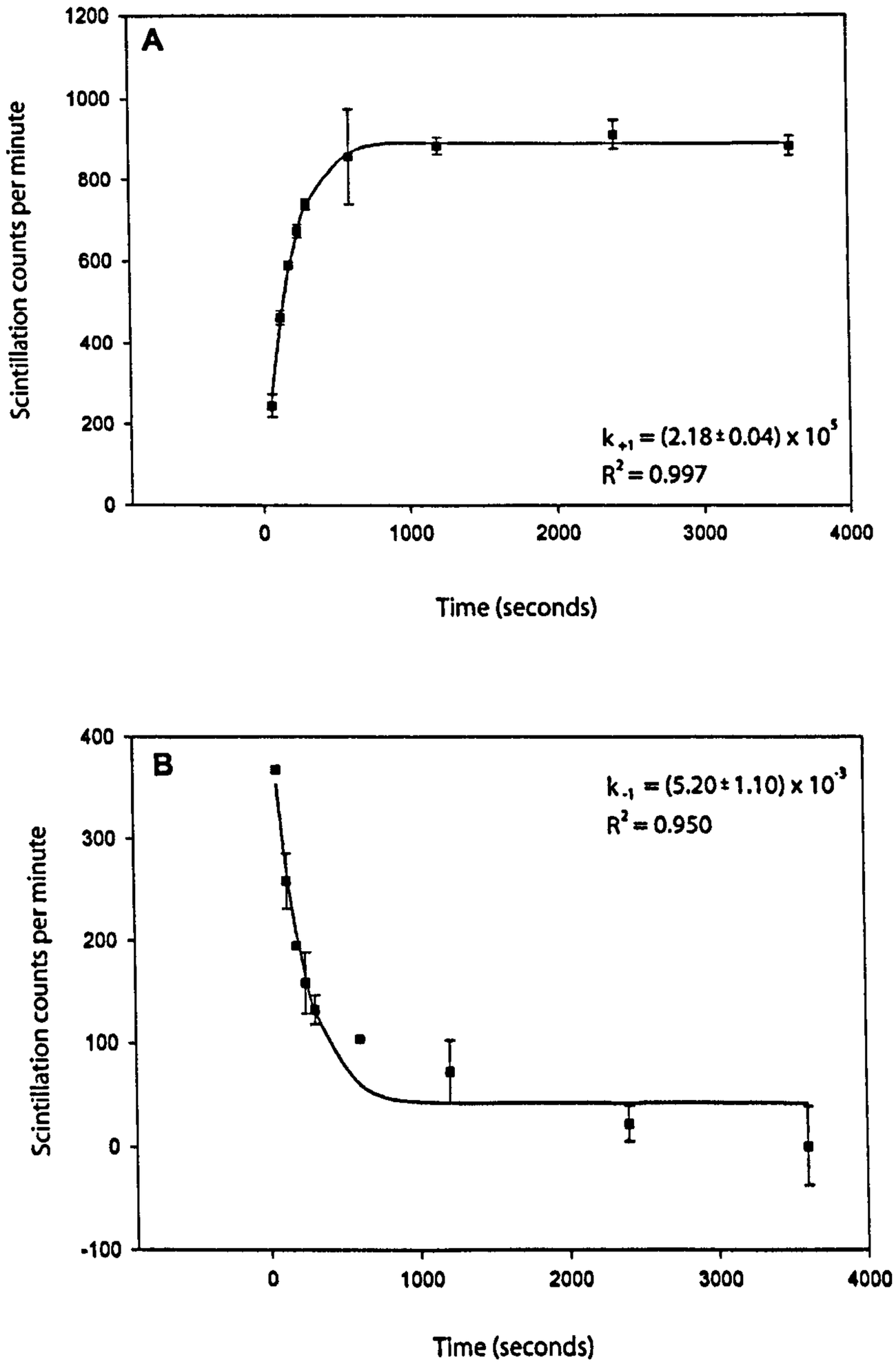

Figure A1.6
Kinetics of IgY-Fc Cυ3-4 [C340S] binding to chicken monocytes

Cell binding assays showing experiments used to determine (A) association rate constant (k_{+1}) and (B) dissociation rate constant (k_{-1}) for recombinant IgY-Fc Cυ3-4 [C340S] binding to MQ-NCSU cells. Assays were performed in duplicate and data is shown as mean ± s.e.m.
In (A) cells were incubated in 10nM ^{125}I-labelled ligand. In (B) cells were first saturated with ^{125}I-labelled ligand then washed and incubated with excess unlabelled ligand. In both experiments, 10^6 cells were removed at the times shown, spun through phthalate oil and their radioactivity measured using an automatic gamma counter. Regression analysis was used to fit the data to non-linear models (see chapter 2). The R^2 value provides a measure of how well the models fit the data, with 1 being a perfect fit.

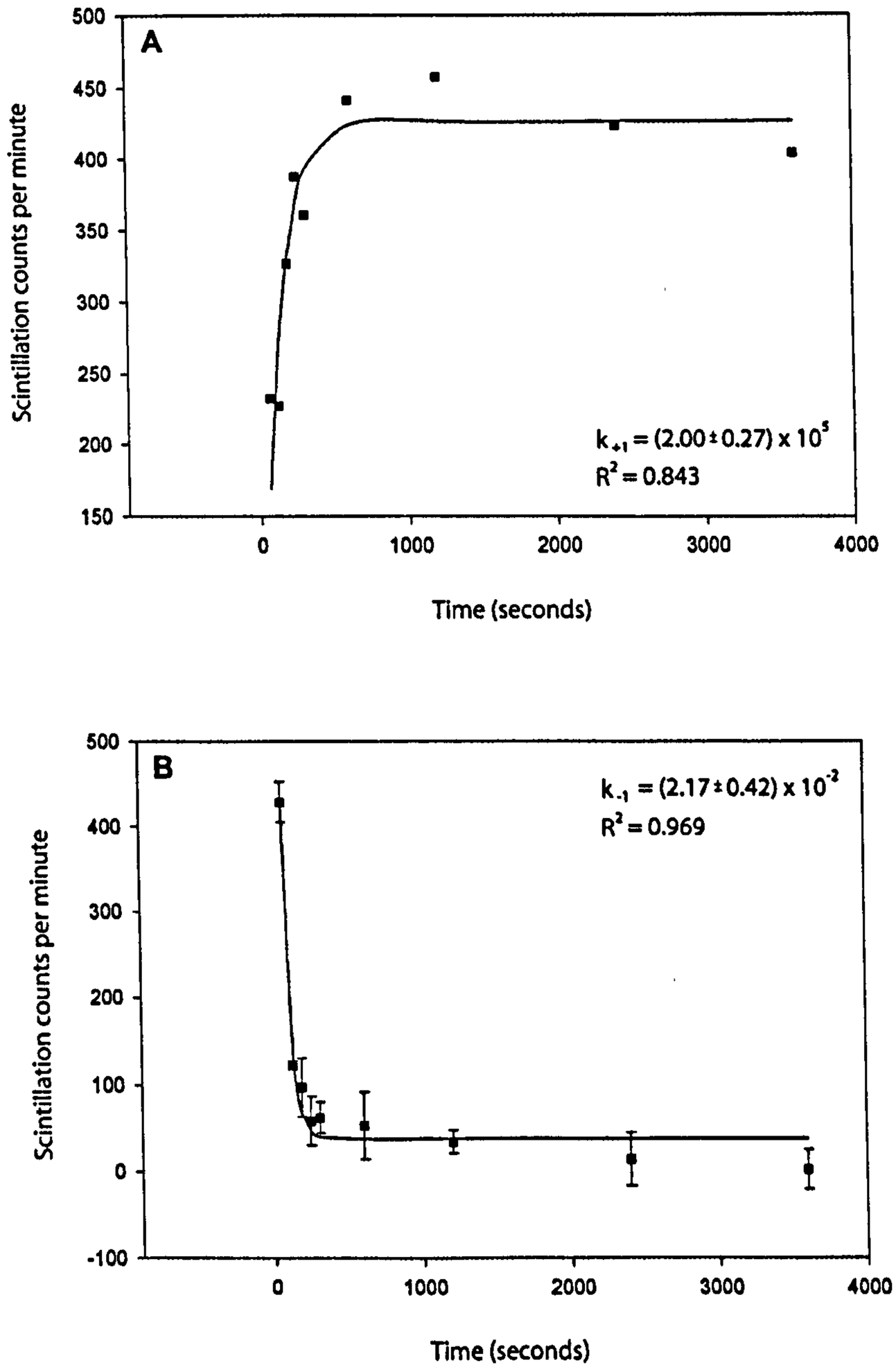

Figure A1.7
Kinetics of IgY-Fc Cυ3-4 [C347S] binding to chicken monocytes

Cell binding assays showing experiments used to determine (A) association rate constant (k_{+1}) and (B) dissociation rate constant (k_{-1}) for recombinant IgY-Fc Cυ3-4 [C347S] binding to MQ-NCSU cells. Assays were performed in duplicate and data is shown as mean ± s.e.m, except for (A) for which a single experiment was performed.
In (A) cells were incubated in 10nM ^{125}I-labelled ligand. In (B) cells were first saturated with ^{125}I-labelled ligand then washed and incubated with excess unlabelled ligand. In both experiments, 10^6 cells were removed at the times shown, spun through phthalate oil and their radioactivity measured using an automatic gamma counter. Regression analysis was used to fit the data to non-linear models (see chapter 2). The R^2 value provides a measure of how well the models fit the data, with 1 being a perfect fit.

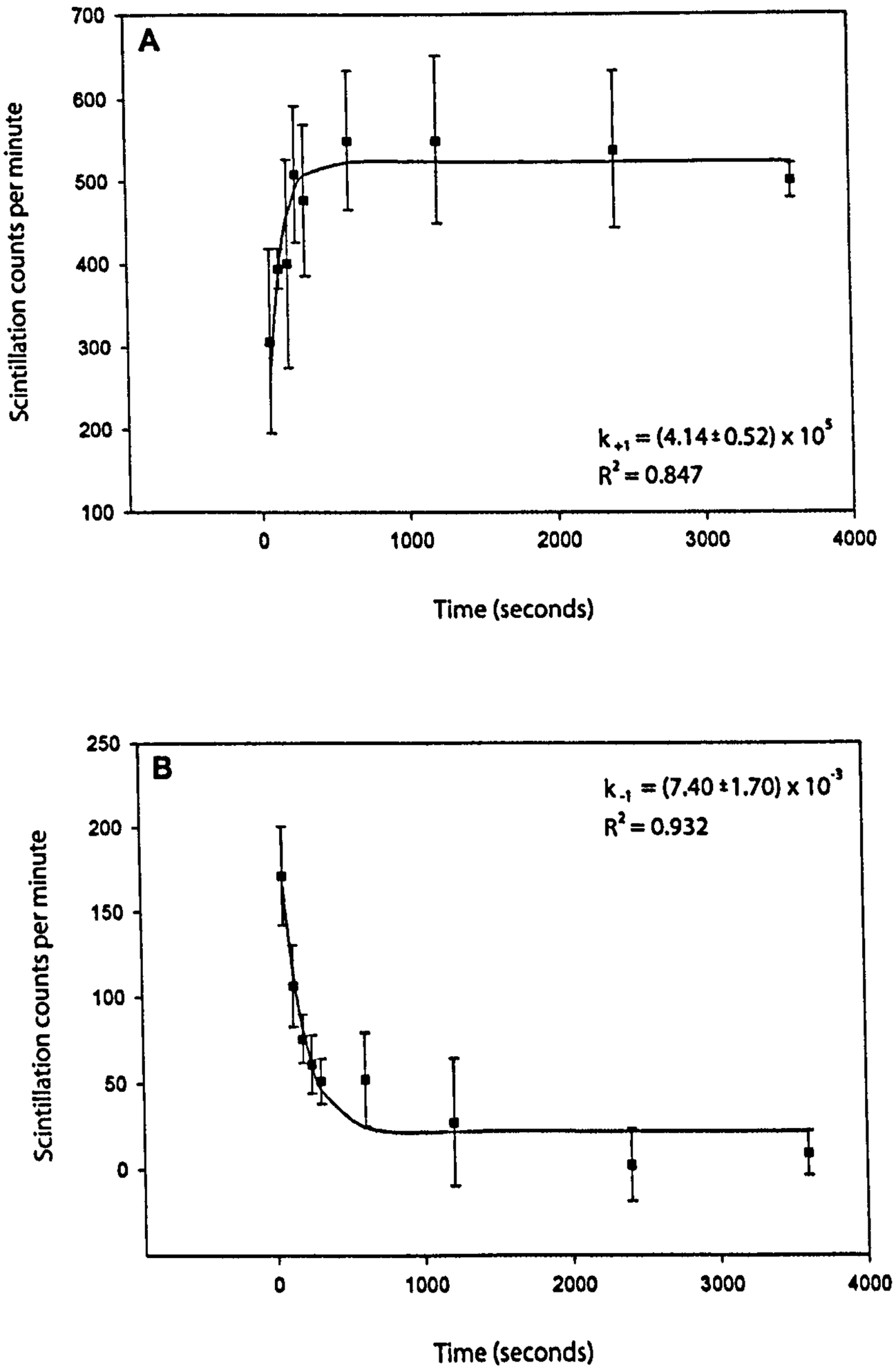

Figure A1.8
Kinetics of IgY-Fc Cυ3-4 [C340S C347S] binding to chicken monocytes

Cell binding assays showing experiments used to determine (A) association rate constant (k_{+1}) and (B) dissociation rate constant (k_{-1}) for recombinant IgY-Fc Cυ3-4 [C340S C347S] binding to MQ-NCSU cells. Assays were performed in duplicate and data is shown as mean ± s.e.m.
In (A) cells were incubated in 10nM ^{125}I-labelled ligand. In (B) cells were first saturated with ^{125}I-labelled ligand then washed and incubated with excess unlabelled ligand. In both experiments, 10^6 cells were removed at the times shown, spun through phthalate oil and their radioactivity measured using an automatic gamma counter. Regression analysis was used to fit the data to non-linear models (see chapter 2). The R^2 value provides a measure of how well the models fit the data, with 1 being a perfect fit.

Fragment	modifications	Species or Expression system	Type	k_{+1}	k_{-1}	K_A	Author (Year)	T	
Fce3-4		mammalian	BIAcore	3.08E+05	6.26E-04	4.92E+08	Hunt (2005)	RT	
Fce3-4	Degly PNGase	mammalian	BIAcore	1.97E+05	1.73E-03	1.14E+08	Hunt (2005)	RT	
Fce3-4	ΔC no interchain S-S	E.coli	BIAcore	2.76E+06	6.63E-02	4.14E+07	Hunt (2005)	RT	2:1 stoic
IgE		human	BIAcore	2.40E+05	1.80E-04	1.33E+09	McDonnell (2001)	RT	
Fce2-4		mammalian	BIAcore	3.00E+05	1.90E-04	1.58E+09	McDonnell (2001)	RT	
Fce3-4		mammalian	BIAcore	9.20E+05	3.40E-03	2.71E+08	McDonnell (2001)	RT	
IgE		human	BIAcore	4.60E+03	1.90E-04	2.42E+07	McDonnell (2001)	RT	CD23
Fce2-4		mammalian	BIAcore	4.80E+03	2.30E-03	2.09E+06	McDonnell (2001)	RT	CD23
Fce3-4		mammalian	BIAcore	1.20E+04	3.20E-03	3.75E+06	McDonnell (2001)	RT	CD23
Fce2-4		mammalian	BIAcore	2.50E+05	6.50E-05	5.40E+09	Henry (1997)	RT	transiently expressed
IgE		human	BIAcore	8.60E+04	3.60E-04	2.40E+08	Henry (1997)	RT	
Fce2-4		mammalian	BIAcore	2.50E+05	2.00E-04	1.20E+09	Henry (1997)	RT	
Fce3-4		mammalian	BIAcore	5.50E+04	1.50E-04	3.70E+08	Henry (1997)	RT	
Fce2-4		mammalian	cell binding (stable cell line)	9.90E+05	1.70E-05	5.70E+10	Henry (1997)	RT?	
Fce2-4		mammalian	cell binding (stable cell line)			3.20E+07	Henry (1997)	RT?	CD23
IgE		human	cell binding (stable cell line)	3.10E+05	9.00E-06	3.40E+10	Young (1995)	37?	
Fce2-4		mammalian	cell binding (stable cell line)	8.10E+05	1.00E-05	8.10E+10	Young (1995)	37?	
Fce2-4	N265Q N371Q	mammalian	cell binding (stable cell line)	9.90E+05	2.00E-05	5.00E+10	Young (1995)	37?	

Fragment	modifications	Species or Expression system	Type	k_{+1}	k_{-1}	K_A	Author (Year)	T	
IgE		human	cell binding (stable cell line)			7.30E+07	Young (1995)	37?	CD23
Fce2-4		mammalian	cell binding (stable cell line)			4.10E+06	Young (1995)	37?	CD23
Fce2-4	N265Q N371Q	mammalian	cell binding (stable cell line)			3.20E+07	Young (1995)	37?	CD23
IgE		human	cell binding (basophils)	3.22E+05	7.18E-05	4.48E+09	Ishizaka (1986)	37	
Fce2-4		E.coli	cell binding (basophils)	3.75E+05	6.58E-05	5.68E+09	Ishizaka (1986)	37	
IgE		rat	cell binding (RBL)	8.80E+04	1.40E-05	6.00E+09	Kulczycki (1974)	37	
IgE		rat	cell binding (RBL)	5.70E+04	4.70E-06	1.20E+10	Kulczycki (1974)	24	
IgE		rat	cell binding (RBL)	2.10E+04	-		Kulczycki (1974)	4	
IgE		human	cell binding (mast cells)	1.90E+05	6.90E-05	2.75E+09	Ogawa (1983)	37	
IgE		human	BIAcore	8.60E+04	3.30E-05	2.70E+09	Cook (1997)	RT	
Fce2-4		mammalian	BIAcore	2.50E+05	7.50E-05	3.30E+09	Cook (1997)	RT	
Fce3-4		mammalian	BIAcore	9.60E+05	6.30E-04	1.50E+09	Cook (1997)	RT	
IgG		human	cell binding (U937)	2.10E+05	1.10E-04	1.90E+09	Shopes (1990)	RT	
IgG		human	cell binding (U937)	2.70E+05	4.50E-04	6.00E+08	Shopes (1995)	37	
IgG		human	cell binding (U937)	9.70E+04	9.00E-06	1.10E+10	Shopes (1995)	4	
IgG		human	cell binding (U937)	1.88E+05	5.50E-05	9.80E+08	Raychaudhuri (1985)	RT	
IgG-Fc		human	cell binding (U937)	6.08E+05	9.50E-05	3.10E+09	Raychaudhuri (1985)	RT	
IgG2a		mouse	cell binding (P388D)		1.40E-03		Unkeless (1975)	4	
IgG2a		mouse	cell binding (P388D)	~10^5	3.50E-03		Unkeless (1975)	RT	
IgG2a		mouse	cell binding (P388D)		4.30E-03		Unkeless (1975)	37	
IgG1		hu/mo chimeric (hu Fc)	BIAcore	1.70E+06	1.80E-04	9.44E+09	Paetz (2005)	RT	
IgG1		human	BIAcore	1.50E+06	3.80E-04	3.95E+09	Paetz (2005)	RT	

Table A1.9 (previous two pages)
Reported binding Fc receptor binding kinetics of IgE, IgG and various Fc fragments

Data reported in the literature from SPR (BIAcore) or cell binding assays of high affinity receptor interactions (FcγRI or FcεRI), unless specified otherwise. T = temperature of experiment, RT = room temp.

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	%ID
1	CU1	105	2	CU2	94	06
1	CU1	105	3	CU3	109	07
1	CU1	105	4	CU4	118	12
1	CU1	105	5	CE1	103	18
1	CU1	105	6	CE2	105	08
1	CU1	105	7	CE3	110	12
1	CU1	105	8	CE4	111	06
1	CU1	105	9	G1-CG1	98	18
1	CU1	105	10	G1-CG2	104	05
1	CU1	105	11	G1-CG3	111	02
1	CU1	105	12	G2-CG1	98	13
1	CU1	105	13	G2-CG2	102	13
1	CU1	105	14	G2-CG3	112	02
1	CU1	105	15	G3-CG1	99	13
1	CU1	105	16	G3-CG2	104	06
1	CU1	105	17	G3-CG3	111	03
1	CU1	105	18	G4-CG1	98	13
1	CU1	105	19	G4-CG2	104	06
1	CU1	105	20	G4-CG3	111	02
2	CU2	94	3	CU3	109	27
2	CU2	94	4	CU4	118	25
2	CU2	94	5	CE1	103	24
2	CU2	94	6	CE2	105	21
2	CU2	94	7	CE3	110	24
2	CU2	94	8	CE4	111	22
2	CU2	94	9	G1-CG1	98	26
2	CU2	94	10	G1-CG2	104	22
2	CU2	94	11	G1-CG3	111	27
2	CU2	94	12	G2-CG1	98	25
2	CU2	94	13	G2-CG2	102	20
2	CU2	94	14	G2-CG3	112	27
2	CU2	94	15	G3-CG1	99	25
2	CU2	94	16	G3-CG2	104	21
2	CU2	94	17	G3-CG3	111	27
2	CU2	94	18	G4-CG1	98	26
2	CU2	94	19	G4-CG2	104	26
2	CU2	94	20	G4-CG3	111	29
3	CU3	109	4	CU4	118	23
3	CU3	109	5	CE1	103	10
3	CU3	109	6	CE2	105	10
3	CU3	109	7	CE3	110	31
3	CU3	109	8	CE4	111	18
3	CU3	109	9	G1-CG1	98	22
3	CU3	109	10	G1-CG2	104	25
3	CU3	109	11	G1-CG3	111	22
3	CU3	109	12	G2-CG1	98	22
3	CU3	109	13	G2-CG2	102	26
3	CU3	109	14	G2-CG3	112	21
3	CU3	109	15	G3-CG1	99	23
3	CU3	109	16	G3-CG2	104	25
3	CU3	109	17	G3-CG3	111	21
3	CU3	109	18	G4-CG1	98	23
3	CU3	109	19	G4-CG2	104	25
3	CU3	109	20	G4-CG3	111	22
4	CU4	118	5	CE1	103	10
4	CU4	118	6	CE2	105	16
4	CU4	118	7	CE3	110	18
4	CU4	118	8	CE4	111	31
4	CU4	118	9	G1-CG1	98	17
4	CU4	118	10	G1-CG2	104	17
4	CU4	118	11	G1-CG3	111	32
4	CU4	118	12	G2-CG1	98	18
4	CU4	118	13	G2-CG2	102	21
4	CU4	118	14	G2-CG3	112	34
4	CU4	118	15	G3-CG1	99	18
4	CU4	118	16	G3-CG2	104	18
4	CU4	118	17	G3-CG3	111	36
4	CU4	118	18	G4-CG1	98	19
4	CU4	118	19	G4-CG2	104	16
4	CU4	118	20	G4-CG3	111	31
5	CE1	103	6	CE2	105	19
5	CE1	103	7	CE3	110	15
5	CE1	103	8	CE4	111	19
5	CE1	103	9	G1-CG1	98	34
5	CE1	103	10	G1-CG2	104	14

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	%ID
5	CE1	103	11	G1-CG3	111	22
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5	CE1	103	13	G2-CG2	102	13
5	CE1	103	14	G2-CG3	112	22
5	CE1	103	15	G3-CG1	99	36
5	CE1	103	16	G3-CG2	104	13
5	CE1	103	17	G3-CG3	111	22
5	CE1	103	18	G4-CG1	98	34
5	CE1	103	19	G4-CG2	104	14
5	CE1	103	20	G4-CG3	111	21
6	CE2	105	7	CE3	110	17
6	CE2	105	8	CE4	111	12
6	CE2	105	9	G1-CG1	98	15
6	CE2	105	10	G1-CG2	104	12
6	CE2	105	11	G1-CG3	111	16
6	CE2	105	12	G2-CG1	98	15
6	CE2	105	13	G2-CG2	102	13
6	CE2	105	14	G2-CG3	112	16
6	CE2	105	15	G3-CG1	99	16
6	CE2	105	16	G3-CG2	104	12
6	CE2	105	17	G3-CG3	111	16
6	CE2	105	18	G4-CG1	98	16
6	CE2	105	19	G4-CG2	104	13
6	CE2	105	20	G4-CG3	111	16
7	CE3	110	8	CE4	111	20
7	CE3	110	9	G1-CG1	98	16
7	CE3	110	10	G1-CG2	104	32
7	CE3	110	11	G1-CG3	111	19
7	CE3	110	12	G2-CG1	98	16
7	CE3	110	13	G2-CG2	102	33
7	CE3	110	14	G2-CG3	112	18
7	CE3	110	15	G3-CG1	99	16
7	CE3	110	16	G3-CG2	104	32
7	CE3	110	17	G3-CG3	111	18
7	CE3	110	18	G4-CG1	98	16
7	CE3	110	19	G4-CG2	104	32
7	CE3	110	20	G4-CG3	111	19
8	CE4	111	9	G1-CG1	98	19
8	CE4	111	10	G1-CG2	104	13
8	CE4	111	11	G1-CG3	111	31
8	CE4	111	12	G2-CG1	98	18
8	CE4	111	13	G2-CG2	102	21
8	CE4	111	14	G2-CG3	112	31
8	CE4	111	15	G3-CG1	99	18
8	CE4	111	16	G3-CG2	104	15
8	CE4	111	17	G3-CG3	111	30
8	CE4	111	18	G4-CG1	98	18
8	CE4	111	19	G4-CG2	104	14
8	CE4	111	20	G4-CG3	111	27
9	G1-CG1	98	10	G1-CG2	104	25
9	G1-CG1	98	11	G1-CG3	111	30
9	G1-CG1	98	12	G2-CG1	98	90
9	G1-CG1	98	13	G2-CG2	102	24
9	G1-CG1	98	14	G2-CG3	112	29
9	G1-CG1	98	15	G3-CG1	99	95
9	G1-CG1	98	16	G3-CG2	104	23
9	G1-CG1	98	17	G3-CG3	111	29
9	G1-CG1	98	18	G4-CG1	98	91
9	G1-CG1	98	19	G4-CG2	104	25
9	G1-CG1	98	20	G4-CG3	111	30
10	G1-CG2	104	11	G1-CG3	111	20
10	G1-CG2	104	12	G2-CG1	98	25
10	G1-CG2	104	13	G2-CG2	102	92
10	G1-CG2	104	14	G2-CG3	112	20
10	G1-CG2	104	15	G3-CG1	99	25
10	G1-CG2	104	16	G3-CG2	104	97
10	G1-CG2	104	17	G3-CG3	111	19
10	G1-CG2	104	18	G4-CG1	98	26
10	G1-CG2	104	19	G4-CG2	104	93
10	G1-CG2	104	20	G4-CG3	111	20
11	G1-CG3	111	12	G2-CG1	98	28
11	G1-CG3	111	13	G2-CG2	102	20
11	G1-CG3	111	14	G2-CG3	112	96
11	G1-CG3	111	15	G3-CG1	99	28
11	G1-CG3	111	16	G3-CG2	104	21

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	%ID
11	G1-CG3	111	17	G3-CG3	111	91
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11	G1-CG3	111	19	G4-CG2	104	22
11	G1-CG3	111	20	G4-CG3	111	94
12	G2-CG1	98	13	G2-CG2	102	24
12	G2-CG1	98	14	G2-CG3	112	27
12	G2-CG1	98	15	G3-CG1	99	93
12	G2-CG1	98	16	G3-CG2	104	23
12	G2-CG1	98	17	G3-CG3	111	27
12	G2-CG1	98	18	G4-CG1	98	95
12	G2-CG1	98	19	G4-CG2	104	25
12	G2-CG1	98	20	G4-CG3	111	28
13	G2-CG2	102	14	G2-CG3	112	20
13	G2-CG2	102	15	G3-CG1	99	24
13	G2-CG2	102	16	G3-CG2	104	93
13	G2-CG2	102	17	G3-CG3	111	19
13	G2-CG2	102	18	G4-CG1	98	25
13	G2-CG2	102	19	G4-CG2	104	92
13	G2-CG2	102	20	G4-CG3	111	20
14	G2-CG3	112	15	G3-CG1	99	27
14	G2-CG3	112	16	G3-CG2	104	21
14	G2-CG3	112	17	G3-CG3	111	95
14	G2-CG3	112	18	G4-CG1	98	27
14	G2-CG3	112	19	G4-CG2	104	23
14	G2-CG3	112	20	G4-CG3	111	94
15	G3-CG1	99	16	G3-CG2	104	23
15	G3-CG1	99	17	G3-CG3	111	27
15	G3-CG1	99	18	G4-CG1	98	95
15	G3-CG1	99	19	G4-CG2	104	25
15	G3-CG1	99	20	G4-CG3	111	28
16	G3-CG2	104	17	G3-CG3	111	20
16	G3-CG2	104	18	G4-CG1	98	24
16	G3-CG2	104	19	G4-CG2	104	92
16	G3-CG2	104	20	G4-CG3	111	21
17	G3-CG3	111	18	G4-CG1	98	27
17	G3-CG3	111	19	G4-CG2	104	22
17	G3-CG3	111	20	G4-CG3	111	90
18	G4-CG1	98	19	G4-CG2	104	26
18	G4-CG1	98	20	G4-CG3	111	28
19	G4-CG2	104	20	G4-CG3	111	23

Table A1.10 (above and previous two pages)
Homology between Ig domains in chicken IgY, human IgE and human IgG1-4

Amino acid sequence identity when individually aligned constant heavy chain Ig domains were from υ (CU1-4), ε (CE1-4) and γ_{1-4} (G_{1-4}-CG1-3). Sequences were aligned using ClustalW (Thompson *et al.*, 1994).

Appendix 2: Published work

Taylor, A. I., Gould, H. J., Sutton, B. J., Calvert, R. A. (2007).

The first avian Ig-like Fc receptor family member combines features of mammalian FcR and FCRL.

Immunogenetics 59 (4), 323-8.

Immunogenetics (2007) 59:323–328
DOI 10.1007/s00251-007-0195-9

BRIEF COMMUNICATION

The first avian Ig-like Fc receptor family member combines features of mammalian FcR and FCRL

Alexander Iain Taylor · Hannah Jane Gould · Brian John Sutton · Rosaleen Ann Calvert

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Abstract Homologues of almost all mammalian Ig-like immunoregulatory receptor families have been found in the chicken, except the Fc receptor (FcR) family. In addition to classical FcRs that specifically bind antibodies and mediate their effector functions, this family includes "Fc receptor-like" (FCRL) proteins for which ligands have yet to be identified. We have cloned and expressed a full-length chicken monocyte transcript that encodes an avian homologue of the mammalian FcR family. We have termed it chFcR/L as it possesses characteristics of both mammalian FcR and FCRL, but is phylogenetically distinct from either. chFcR/L is a transmembrane protein with four extracellular Ig-like domains and a short cytoplasmic tail. It can be expressed on the cell surface only in the presence of an accessory molecule, chFcRγ, through which it acquires signalling potential.

Keywords Chicken · Avian · Fc receptor · Fc receptor homologue · FcR · FCRL

In the vertebrate adaptive immune system, the location and nature of the response to antigenic challenge depends on the production of different antibody isotypes and the expression of corresponding Fc receptors (FcR) on a variety of cell types (Ravetch and Kinet 1991). Most FcRs are members of the immunoglobulin (Ig) superfamily. The α chains of the FcR for the IgG subclasses, FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16) and FcγRIV, and the high affinity receptor for IgE, FcεRI, are composed of two or three closely related extracellular Ig-like domains that contain the Fc-binding site, a transmembrane domain and a cytoplasmic tail. Except for FcγRII and its isoforms, the FcRα chains invariably lack signalling motifs in their cytoplasmic tails and must associate with adaptor molecules (typically FcεRIγ) to transduce signals.

Data mining of genomes and expressed sequence tag (EST) libraries has shown that all immunoreceptor families, of which FcR is one, show considerable diversity. Numerous genes have been identified in mice and humans that have genomic proximity and homology to the classical FcR and are collectively described as 'FcR-like' (FCRL). Despite the presence of domains closely related to those responsible for Fc binding in FcγRI, FcγRII, FcγRIII and FcεRI, the FCRL appear to lack the ability to bind Fc (Davis et al. 2002). As yet, no ligands have been found for any FCRL, but their preferential expression on B cells (Davis et al. 2001; Hatzivassiliou et al. 2001; Miller et al. 2002) and the presence of one or more immunoreceptor tyrosine-based activation (ITAM) or inhibitory (ITIM) signalling motifs, which have been shown to be functional (Ehrhardt et al. 2003; Leu et al. 2005), is consistent with an immunoregulatory role. A link between polymorphisms in an *FCRL* and autoimmune disease is also suggestive of such a function (Ikari et al. 2006; Kochi et al. 2005; Umemura et al. 2006).

FCRL sequences have been identified in bony fish and an amphibian, vertebrate classes that are phylogenetically distant from mammals (Davis et al. 2005; Stafford et al. 2006). To date, no direct homologues of this family of immune receptors have been found in birds. The chicken (*Gallus gallus*) is an invaluable model organism for comparative studies, as it allows us to study the evolutionary link between mammals and more basal vertebrates.

The EMBL accession number of the nucleotide sequence reported here is AM412311.

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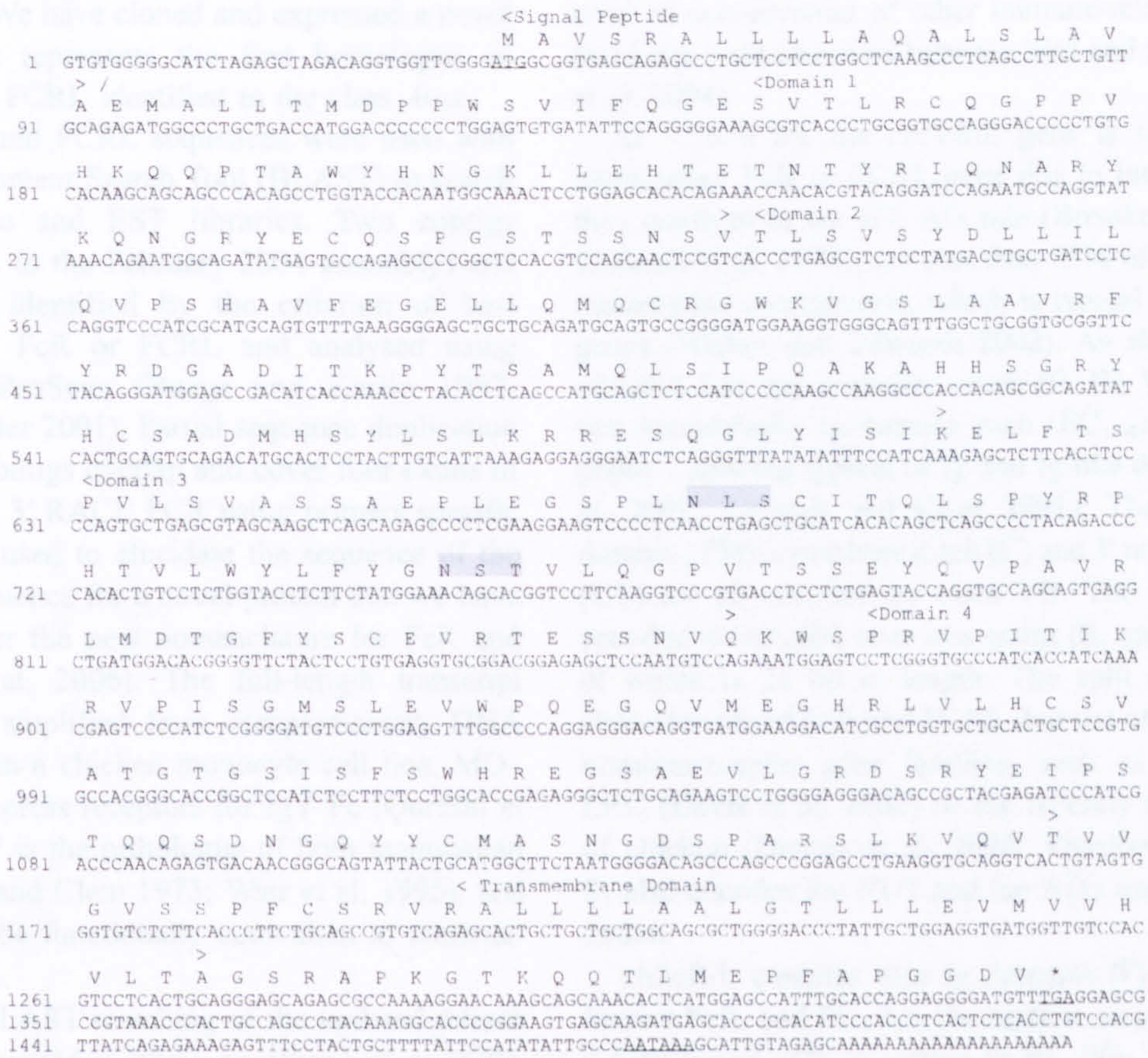


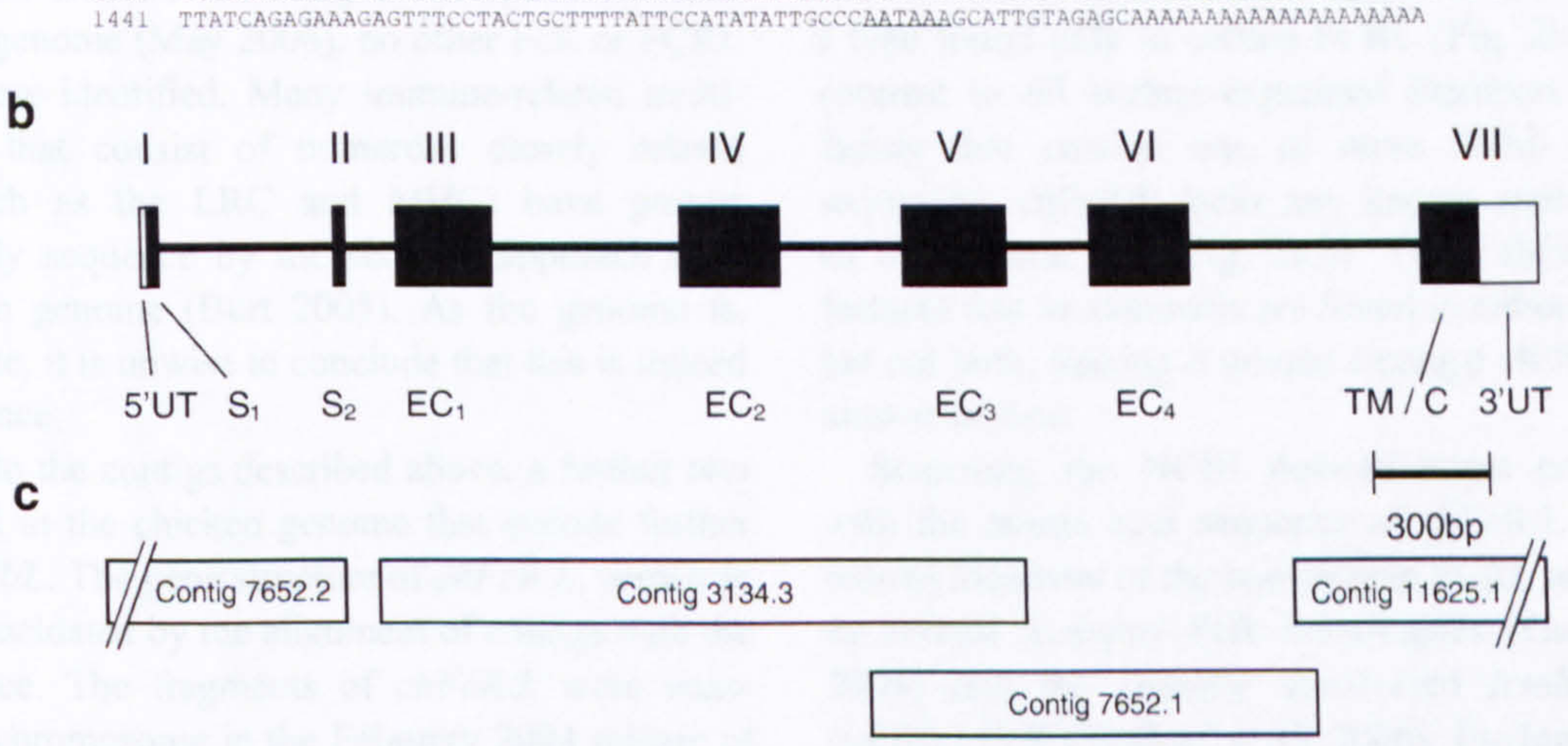


Fig. 1 Sequence characteristics of chicken chFcR/L. **a** Nucleotide and predicted amino acid sequence of chFcR/L. The full length cDNA was cloned by SMART RACE–PCR (Clontech, UK) using primers based on sequences homologous to mammalian FcR and FCRL identified in the chicken genome (http://www.ensembl.org/Gallus_gallus/index.html) and BBSRC chickEST database (http://www.chick.umist.ac.uk/). Protein motifs were identified using InterProScan (Zdobnov and Apweiler 2001). Predicted signal peptide, Ig domains and transmembrane helix are labelled above the amino acid sequence. The signal peptide cleavage site, marked with a *slash*, was predicted to be between residues 19 (A) and 20 (E) using SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/). Potential *N*-glycosylation sites are shaded *grey*; polyadenylation sequence is *underlined* and the stop codon (TGA) is *underlined* and marked with an *asterisk*). Nucleotide numbers are shown at the *left*. **b** Schematic *chFcR/L* gene representation. Contigs covering almost all of the *chFcR/L* gene were identified in the chicken genome by the BLAST search using the chFcR/L cDNA sequence. PCR using genomic DNA was used to corroborate the contigs and to complete the sequence. Exons are numbered and labelled according to the regions they encode (*filled* indicates translated sequence): *5'UT* and *3'UT* untranslated regions, *S1* and *S2* signal peptide, EC_{1-4} extracellular Ig domains 1–4, *TM* transmembrane helix, *C* cytoplasmic tail. **c** Realignment of *chFcR/L* in the chicken genome. The correct order of contigs that cover *chFcR/L*, labelled with their 2004 assembly ID codes. The EMBL accession number for chFcR/L cDNA is AM412311

(Hillier et al. 2004). We have cloned and expressed a novel chicken protein that represents the first homologue of mammalian FcR and FCRL identified in the class *Aves*.

Mammalian FcR and FCRL sequences were used with the Basic Local Alignment Search Tool (BLAST) to search the chicken genome and EST libraries. Two contigs (7,652.1 and 3,134.3 in the February 2004 assembly) and several ESTs were identified by the criterion of best reciprocal hit to an FcR or FCRL and analysed using Genscan and InterProScan (Burge and Karlin 1997; Zdobnov and Apweiler 2001). Partial sequence duplication suggests that these contigs overlap and cover four exons of a larger gene. 5′ and 3′ RACE PCR using primers specific for each exon were used to elucidate the sequence of the complete coding sequence for a novel protein that we have termed chFcR/L after the new nomenclature for FcR and FCRL (Maltais et al. 2006). The full-length transcript (Fig. 1a) was then amplified from complementary DNA (cDNA) derived from a chicken monocyte cell line, MQ–NCSU, known to express receptors for IgY Fc (Qureshi et al. 1990). Avian IgY is the orthologue of both mammalian IgG and IgE (Faith and Clem 1973; Warr et al. 1995), and thus, its FcRs may be functionally equivalent to mammalian FcγR and FcεR.

Despite further BLAST searching of the updated release of the chicken genome (May 2006), no other FcR or FCRL homologues were identified. Many immune-related multigene families that consist of numerous closely related sequences (such as the LRC and MHC) have proven difficult to fully sequence by the shotgun approach used for the chicken genome (Burt 2005). As the genome is, thus, incomplete, it is unwise to conclude that this is indeed the only sequence.

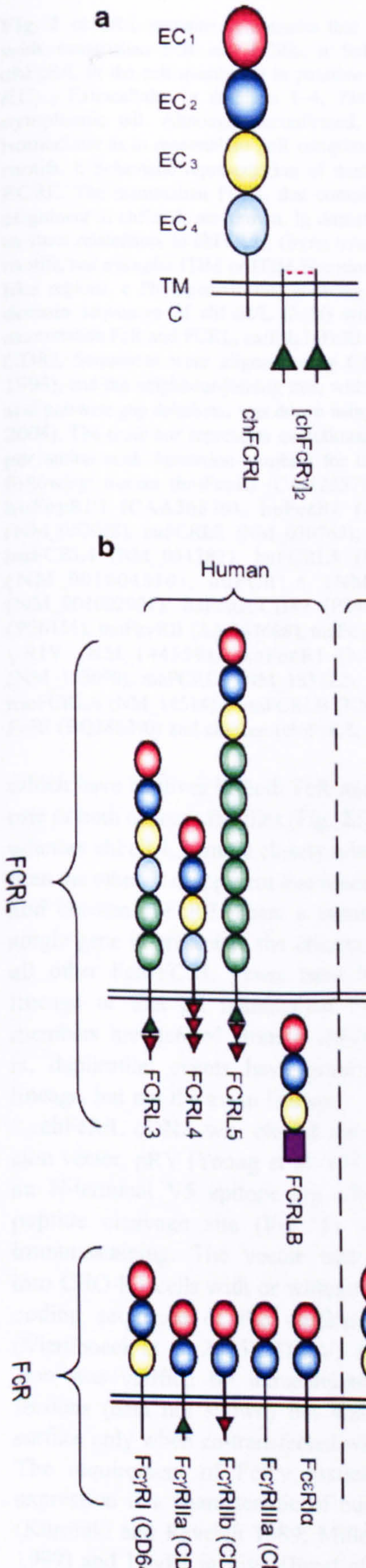
In addition to the contigs described above, a further two were identified in the chicken genome that encode further exons of *chFcR/L*. The gene structure of *chFcR/L*, shown in Fig. 1b, was elucidated by the alignment of contigs with the cDNA sequence. The fragments of *chFcR/L* were unassigned to any chromosome in the February 2004 release of the chicken genome, but have since been anchored to microchromosome 25 in the March 2006 assembly. In both releases, *chFcR/L* is incorrectly assembled, as it lies in a region of Ig domains that are known to be difficult to order, but we have now definitively realigned the relevant contigs in the chicken genome (Fig. 1c).

The full length chFcR/L cDNA contains a 1,308-bp coding sequence for a 20aa signal peptide and a 415aa transmembrane glycoprotein consisting of four extracellular domains that belong to the immunoglobulin (Ig) superfamily, a transmembrane helix and a short cytoplasmic tail (Fig. 1a). The extracellular Ig domains have up to 48% amino acid identity with Ig domains of the members of the mammalian FcR and FCRL, which is consistent with the level of conservation of other immune-related orthologues that have been observed between bird and mammal (Hillier et al. 2004).

At ~3,670 bp, the *chFcR/L* gene is smaller than any mammalian FcR or FCRL gene due to introns that, whilst they conform to the GT–AG rule (Breathnach et al. 1978; Catterall et al. 1978), are less than 25% of the size of their mammalian counterparts, which is typical of most chicken genes (Waltari and Edwards 2002). As shown in Fig. 1b, *chFcR/L* has seven exons: exons III, IV, V and VI encode one extracellular Ig domain each (EC_{1-4}), and follow the phase 1 splicing typical of Ig and Ig-like domains (Davis et al. 2002; Ravetch and Kinet 1991). The transmembrane domain (TM), cytoplasmic tail (C) and 3′ untranslated region (UT) are all encoded by exon VII. The signal peptide is encoded in *chFcR/L* over two exons (S_1 and S_2), the second of which is 21 bp in length. The split signal peptide is characteristic of FcR and FCRL, but not other Ig-containing immunoreceptor gene families, such as the mammalian LRC (Davis et al. 2002) or the recently discovered CHIR of chicken (Dennis et al. 2000; Viertlboeck et al. 2005). S_1 also encodes the 5′UT and the ATG translation initiation codon.

chFcR/L contains four Ig domains (Fig. 2a) unlike any known FcR, and EC_4 has the highest sequence similarity to a type found only in certain FCRL (Fig. 2b). However, in contrast to all surface-expressed members of the FCRL family that contain one or more ITIM- or ITAM-like sequences, chFcR/L lacks any known signalling motif in its cytoplasmic tail (Fig. 2a,b). Thus, chFcR/L combines features that in mammals are found in either FcR or FCRL, but not both, making it unique amongst all family members known to date.

Screening the NCBI non-redundant protein database with the amino acid sequence of chFcR/L using BLAST returns members of the mammalian FCRL and FcR, as well as several *Xenopus* FcR homologues (Guselnikov et al. 2004) and the recently discovered *Ictalurus punctatus* (catfish) FcR (Stafford et al. 2006). Phylogenetic analysis, comparing these hits with individual domains of chFcR/L, reveals that every domain has a related counterpart that variously occurs throughout the FcR and FCRL (coloured according to homology in Fig. 2a–c). A single homologous domain type is primarily responsible for Ig binding in each of the FcR (coloured dark blue in Fig. 2b). Domain EC_2 of chFcR/L shares greatest homology with this domain, and it contains the conserved residues that are its hallmark (Davis et al. 2002). However, it is difficult to resolve where chFcR/L fits into the phylogeny of the FcR and FCRL. Profuse exon duplication events have occurred in a lineage-restricted fashion, and the evolutionary distance between mammals, birds, amphibians and fish has led to the considerable divergence of immune-related genes. Domains EC_1–EC_3,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FcγRIII (CD16)
FcγRIV
FcγRIIb (CD32)
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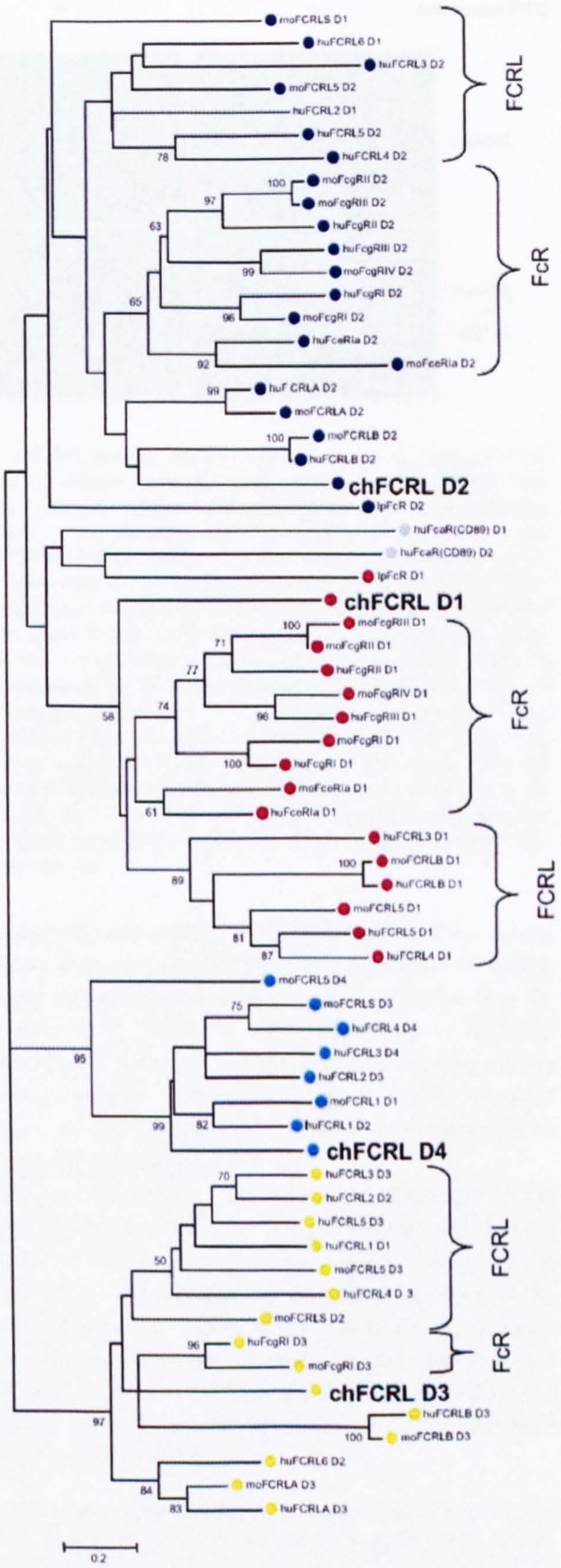
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0.2

◀ **Fig. 2** chFcR/L contains Ig domains that share a common ancestry with mammalian FcR and FCRL. **a** Schematic representation of chFcR/L in the cell membrane in putative association with chFcRγ. EC_{1-4} Extracellular Ig domains 1–4, *TM* transmembrane helix, *C* cytoplasmic tail. Although unconfirmed, chFcRγ is shown as a homodimer as in mammalian FcR complexes. *Green triangles* ITAM motifs. **b** Schematic representation of mammalian FcR and selected FCRL. The mammalian FCRL that contain the longest stretches of alignment to chFcR/L are shown. Ig domains are coloured according to their relatedness to chFcR/L. *Green triangles* ITAM or ITAM-like motifs, *red triangles* ITIM or ITIM-like motifs, *purple squares* mucin-like regions. **c** Phylogenetic tree showing comparison of individual domain sequences of chFcR/L (*bold*) with those of representative mammalian FcR and FCRL, catfish IpFcRI and a member of the LRC, CD89. Sequences were aligned using ClustalW (Thompson et al. 1994), and the neighbour-joining tree, with 1,000 bootstrap replicates and pairwise gap deletions, was drawn using MEGA 3.1 (Kumar et al. 2004). The *scale bar* represents evolutionary distance in substitutions per amino acid. Accession numbers for the sequences used are the following: human (hu)FcγRI (CAI12557), huFcγRII (CAA35642), huFcγRIII (CAA36870), huFcεRI (AAH05912), huFCRL1 (NM_052938), huFCRL2 (NM_030764), huFCRL3 (NM_052939), huFCRL4 (NM_031282), huFCRL5 (NM_031281), huFCRL6 (NM_001004310), huFCRLA (NM_032738), huFCRLB (NM_001002901), huFcαR/CD89 (P24071), mouse (mo)FcγRI (P26151), moFcγRII (AAA37608), moFcγRIII (NP_034318), moFcγRIV (NM_144559), moFcεRI (NP_034314), moFCRL1 (NM_153090), moFCRL5 (NM_183222), moFCRLS (NM_030707), moFCRLA (NM_145141), moFCRLB (NM_001029984), catfish (Ip)FcRI (DQ286290) and chicken (ch)FcR/L (AM412311)

which have relatives in both FcR and FCRL, are outliers to one or both of these families (Fig. 2c). Although it is unclear whether chFcR/L is more closely related to one family rather than the other, it is apparent that mammalian FcR and FCRL, and chicken chFcR/L share a common ancestry. If just a single gene is present in the chicken, it suggests either that all other FcR/FCRL genes have been lost in the avian lineage or that all mammalian FcR and FCRL family members are derived from a chFcR/L-like ancestor—that is, duplication events have occurred in the mammalian lineage, but not the avian lineage.

chFcR/L cDNA was cloned into a mammalian expression vector, pRY (Young et al. 1995), with the addition of an N-terminal V5 epitope tag after the predicted signal peptide cleavage site (Fig. 1) to allow detection by immunostaining. The vector was transiently transfected into CHO-K1 cells with or without a vector containing the coding sequence of the chicken orthologue of FcRγ (Viertlboeck et al. 2005). The V5 tagged chFcR/L expression was verified by intracellular staining in all transfections (data not shown) but was detectable at the cell surface only when co-transfected with the chFcRγ (Fig. 3). The requirement of FcRγ association for cell surface expression is a characteristic of human FcεRIα, FcγRIIIa (Kurosaki and Ravetch 1989; Miller et al. 1989; Ra et al. 1989) and FcγRI in vivo (Ernst et al. 1993; Masuda and Roos 1993; van Vugt et al. 1996), although the latter can be transiently expressed in its absence (Allen and Seed 1989).

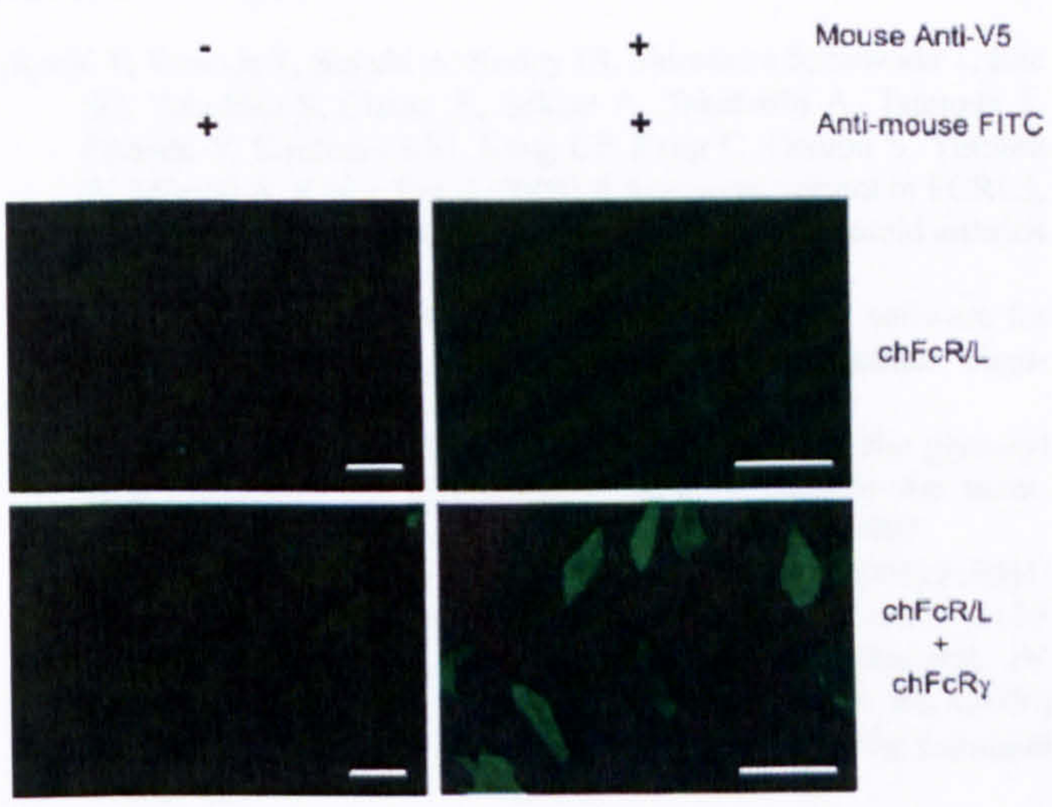

Fig. 3 chFcR/L protein can be expressed at the cell surface in the presence of chFcRγ. CHO-K1 cells were grown in DMEM 10% FCS on chamber slides and transfected with a mammalian expression vector encoding chFcR/L with an N-terminal V5 tag (GKPIPNPLLGLDST) and a 5′ leader sequence from the variable kappa light-chain of the mouse monoclonal antibody B72.3, either alone (*top*) or in conjunction with a similar vector encoding chFcRγ (*bottom*), using TransIt CHO transfection reagent (Mirus Bio, USA), as per the manufacturer's protocol. Cells were fixed with 4% paraformaldehyde in PBS and stained with either mouse anti-V5 (Sigma-Aldrich, USA) at 1 μg/ml followed by anti-mouse FITC (Dako, UK) at 10 μg/ml (*right*) or with anti-mouse FITC alone (*left*). Cells were washed with PBS containing 1% BSA three times for 15 min each between all steps and incubated with antibodies in the same buffer for 1 h at 4°C. FITC was detected and photographed using a Zeiss fluorescence Axioscope fitted with an AxioCam CCD. Scale bar=50 μm

In contrast, the mammalian FCRL that contain TMs do not require an accessory molecule for their expression or ability to trigger intracellular signalling cascades (Ehrhardt et al. 2003; Leu et al. 2005). In vitro expression of chFcR/L confirms that it is indeed a transmembrane protein, and its apparent association with an orthologue of FcRγ suggests that it is an activating receptor. Work is in progress to investigate IgY binding to chFcR/L.

To date, orthologues that represent almost all of the mammalian Ig-like immunoreceptor families have been described in the chicken, with the notable absence of the FcR and FCRL (Viertlboeck et al. 2005). chFcR/L is the first member of this family to be identified in an avian species. Its unique features and phylogenetic relationship to key players in the mammalian immune system makes it a significant addition to our knowledge of this important immunoregulatory family.

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References

Allen JM, Seed B (1989) Isolation and expression of functional high-affinity Fc receptor complementary DNAs. Science 243:378–381

Breathnach R, Benoist C, O'Hare K, Gannon F, Chambon P (1978) Ovalbumin gene: evidence for a leader sequence in mRNA and DNA sequences at the exon–intron boundaries. Proc Natl Acad Sci USA 75:4853–4857

Burge C, Karlin S (1997) Prediction of complete gene structures in human genomic DNA. J Mol Biol 268:78–94

Burt DW (2005) Chicken genome: current status and future opportunities. Genome Res 15:1692–1698

Catterall JF, O'Malley BW, Robertson MA, Staden R, Tanaka Y, Brownlee GG (1978) Nucleotide sequence homology at 12 intron–exon junctions in the chick ovalbumin gene. Nature 275:510–513

Davis RS, Wang YH, Kubagawa H, Cooper MD (2001) Identification of a family of Fc receptor homologs with preferential B cell expression. Proc Natl Acad Sci USA 98:9772–9777

Davis RS, Dennis G Jr, Odom MR, Gibson AW, Kimberly RP, Burrows PD, Cooper MD (2002) Fc receptor homologs: newest members of a remarkably diverse Fc receptor gene family. Immunol Rev 190:123–136

Davis RS, Ehrhardt GR, Leu CM, Hirano M, Cooper MD (2005) An extended family of Fc receptor relatives. Eur J Immunol 35:674–680

Dennis G Jr, Kubagawa H, Cooper MD (2000) Paired Ig-like receptor homologs in birds and mammals share a common ancestor with mammalian Fc receptors. Proc Natl Acad Sci USA 97:13245–13250

Ehrhardt GR, Davis RS, Hsu JT, Leu CM, Ehrhardt A, Cooper MD (2003) The inhibitory potential of Fc receptor homolog 4 on memory B cells. Proc Natl Acad Sci USA 100:13489–13494

Ernst LK, Duchemin AM, Anderson CL (1993) Association of the high-affinity receptor for IgG (Fc gamma RI) with the gamma subunit of the IgE receptor. Proc Natl Acad Sci USA 90:6023–6027

Faith RE, Clem LW (1973) Passive cutaneous anaphylaxis in the chicken. Biological fractionation of the mediating antibody population. Immunology 25:151–164

Guselnikov SV, Erilova A, Najakshin AM, Cohen N, Robert J, Taranin AV (2004) FcR-like genes in the amphibian Xenopus: species-specific expansion, structural diversity, and developmental expression. 12th Int Cong Immunol and 4th Annu Conf FOCIS, Montreal, Abstract 2881

Hatzivassiliou G, Miller I, Takizawa J, Palanisamy N, Rao PH, Iida S, Tagawa S, Taniwaki M, Russo J, Neri A, Cattoretti G, Clynes R, Mendelsohn C, Chaganti RS, Dalla-Favera R (2001) IRTA1 and IRTA2, novel immunoglobulin superfamily receptors expressed in B cells and involved in chromosome 1q21 abnormalities in B cell malignancy. Immunity 14:277–289

Hillier LW, Miller W, Birney E, Warren W, Hardison RC, Ponting CP, Bork P, Burt DW, Groenen MA, Delany ME, Dodgson JB, Chinwalla AT, Cliften PF, Clifton SW, Delehaunty KD, Fronick C, Fulton RS, Graves TA, Kremitzki C, Layman D et al (2004) Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature 432:695–716

Ikari K, Momohara S, Nakamura T, Hara M, Yamanaka H, Tomatsu T, Kamatani N (2006) Supportive evidence for a genetic association of the FCRL3 promoter polymorphism with rheumatoid arthritis. Ann Rheum Dis 65:671–673

Kochi Y, Yamada R, Suzuki A, Harley JB, Shirasawa S, Sawada T, Bae SC, Tokuhiro S, Chang X, Sekine A, Takahashi A, Tsunoda T, Ohnishi Y, Kaufman KM, Kang CP, Kang C, Otsubo S, Yumura W, Mimori A, Koike T et al (2005) A functional variant in FCRL3, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities. Nat Genet 37:478–485

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Kurosaki T, Ravetch JV (1989) A single amino acid in the glycosyl phosphatidylinositol attachment domain determines the membrane topology of Fc gamma RIII. Nature 342:805–807

Leu CM, Davis RS, Gartland LA, Fine WD, Cooper MD (2005) FcRH1: an activation coreceptor on human B cells. Blood 105:1121–1126

Maltais LJ, Lovering RC, Taranin AV, Colonna M, Ravetch JV, Dalla-Favera R, Burrows PD, Cooper MD, Davis RS (2006) New nomenclature for Fc receptor-like molecules. Nat Immunol 7:431–432

Masuda M, Roos D (1993) Association of all three types of Fc gamma R (CD64, CD32, and CD16) with a gamma-chain homodimer in cultured human monocytes. J Immunol 151:7188–7195

Miller L, Blank U, Metzger H, Kinet JP (1989) Expression of high-affinity binding of human immunoglobulin E by transfected cells. Science 244:334–337

Miller I, Hatzivassiliou G, Cattoretti G, Mendelsohn C, Dalla-Favera R (2002) IRTAs: a new family of immunoglobulinlike receptors differentially expressed in B cells. Blood 99:2662–2669

Qureshi MA, Miller L, Lillehoj HS, Ficken MD (1990) Establishment and characterization of a chicken mononuclear cell line. Vet Immunol Immunopathol 26:237–250

Ra C, Jouvin MH, Blank U, Kinet JP (1989) A macrophage Fc gamma receptor and the mast cell receptor for IgE share an identical subunit. Nature 341:752–754

Ravetch JV, Kinet JP (1991) Fc receptors. Annu Rev Immunol 9:457–492

Stafford JL, Wilson M, Nayak D, Quiniou SM, Clem LW, Miller NW, Bengten E (2006) Identification and characterization of a FcR homolog in an ectothermic vertebrate, the channel catfish (*Ictalurus punctatus*). J Immunol 177:2505–2517

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Umemura T, Ota M, Hamano H, Katsuyama Y, Kiyosawa K, Kawa S (2006) Genetic association of Fc receptor-like 3 polymorphisms with autoimmune pancreatitis in Japanese patients. Gut 55:1367–1368

van Vugt MJ, Heijnen AF, Capel PJ, Park SY, Ra C, Saito T, Verbeek JS, van de Winkel JG (1996) FcR gamma-chain is essential for both surface expression and function of human Fc gamma RI (CD64) in vivo. Blood 87:3593–3599

Viertlboeck BC, Habermann FA, Schmitt R, Groenen MA, Du Pasquier L, Gobel TW (2005) The chicken leukocyte receptor complex: a highly diverse multigene family encoding at least six structurally distinct receptor types. J Immunol 175:385–393

Waltari E, Edwards SV (2002) Evolutionary dynamics of intron size, genome size, and physiological correlates in Archosaurs. Am Nat 160:539–552

Warr GW, Magor KE, Higgins DA (1995) IgY: clues to the origins of modern antibodies. Immunol Today 16:392–398

Young RJ, Owens RJ, Mackay GA, Chan CM, Shi J, Hide M, Francis DM, Henry AJ, Sutton BJ, Gould HJ (1995) Secretion of recombinant human IgE-Fc by mammalian cells and biological activity of glycosylation site mutants. Protein Eng 8:193–199

Zdobnov EM, Apweiler R (2001) InterProScan—an integration platform for the signature-recognition methods in InterPro. Bioinformatics 17:847–848